VITAMIN A AND INTRAMUSCULAR FAT DEPOSITION: A NUTRIGENETIC INVESTIGATION IN BEEF CATTLE

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Saskatoon, SK Canada

By

ALISON KATHERINE WARD

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ABSTRACT

Vitamin A restriction has been associated with increased marbling in beef cattle. The purpose of this study was to investigate a possible nutrigenetic mechanism leading to this increase in intramuscular fat. Four genes involved in the vitamin A pathway were examined for genetic polymorphisms that could alter mRNA expression or protein structure. A total of fourteen SNPs were found in bovine *alcohol dehydrogenase 1C* (*ADH1C*), *aldehyde dehydrogenase 1A1* (*ALDH1A1*), *peroxisome proliferator-activated receptor γ* (*PPARγ*), and *retinol binding protein 4* (*RBP4*). Five of these SNPs were examined for associations with production and carcass traits in a previously reported population of 400 crossbred steers. *ALDH1A1 c.*27C>T* was significantly associated with backgrounding average daily gain ($P<0.05$) however no associations were found between *ADH1C c.-64T>C*, *ADH1C c.967C>T*, *ALDH1A1 c.*109A>G*, or *PPARγ c.1344G>T* with any of the parameters measured.

A nutritional study was performed to examine the interaction between *ADH1C c.-64T>C* and vitamin A restriction on production and carcass traits in cattle. It was hypothesized that a phenotypic effect would be observed only when vitamin A was restricted. An initial population of 450 black Angus cross steers were genotyped at *ADH1C c.-64T>C* and from that population 130 steers (50 TT, 50 CT, and 30 CC) were randomly selected for use in the vitamin A restriction study. They were backgrounded for three months on a low β-carotene diet and finished for five months on a diet that did not contain supplemental vitamin A. During the finishing period the steers were treated with monthly boluses of either 0 IU (unsupplemented) or 750,000 IU (supplemented) supplemental vitamin A. Liver and fat biopsies were obtained at the start and end of finishing from a subgroup of five steers per genotype per treatment to assess vitamin A status and measure gene expression. Marbling score was significantly greater ($P<0.05$) in unsupplemented steers. A significant interaction was found between genotype and vitamin A supplementation on intramuscular fat content. Within the unsupplemented treatment, TT steers had significantly greater intramuscular fat than CC steers. Within TT steers, unsupplemented steers had significantly greater intramuscular fat than supplemented animals. Expression of *ADH1C* in the liver was significantly higher in TT steers than CC steers. *ADH1C c.-64T>C* in combination with reduced vitamin A supplementation could potentially be implemented in marker-assisted management to maximize intramuscular fat deposition in finishing cattle.

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LIST OF ABBREVIATIONS

ADG	Average daily gain
ADH1C	Alcohol dehydrogenase 1C
ALDH1A1	Aldehyde dehydrogenase 1A1
ANOVA	Analysis of variance
bp	Base pair
BTA	*Bos taurus* autosome
C/EBP	CCAAT/enhancer binding protein
cDNA	Complimentary deoxyribonucleic acid
CRABP1	Cellular retinoic acid binding protein 1
CRABP 2	Cellular retinoic acid binding protein 2
CRBP	Cellular retinol binding protein
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FABP4	Fatty acid binding protein 4
IGF-1	Insulin-like growth factor 1
IMF	Intramuscular fat content
IU	International unit
LSD	Least significant difference
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PPARγ	Peroxisome proliferator-activated receptor γ
Pu	Purine
qPCR	Quantitative polymerase chain reaction
RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RBP4	Retinol binding protein 4
RE	Retinol equivalents

REA	Retinol activity equivalents
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RXR	Retinoid X receptor
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
STRA6	Stimulated by retinoic acid 6
TTR	Transthyrine
USDA	United Stated Department of Agriculture
UTR	Untranslated region

1 INTRODUCTION

Marker-assisted management incorporates genetic markers into animal management, applying different treatments (*e.g.* diet, days on feed, implant use) to different genotypes to maximize profitability. An example currently implemented in the cattle industry is the use of *leptin R25C* by commercial feedlots. Cattle that are TT at this SNP reach the same backfat thickness on average 16 days sooner than CC cattle (Buchanan *et al*. 2007). Feedlots sort cattle by genotype and finish TT cattle for fewer days on feed than CT or CC cattle, thereby improving uniformity and reducing feed costs from overfinishing (Furber 2011).

Marker-assisted management is an application of nutrigenetics, the study of how genetics and nutrition interact to affect phenotype. Vitamin A and genes within the vitamin A pathway warrant nutrigenetic investigation as vitamin A restriction has been demonstrated to increase marbling in beef cattle (Gorocica-Buenfil *et al*. 2007a).

Marbling is the visible intramuscular fat within a cross-section of meat. It is positively correlated with juiciness, perceived tenderness, and palatability of beef (Wheeler *et al*. 1994). Quality grade, which is a measurement of marbling, has a significant impact on carcass price when cattle are marketed on a grid system. Higher quality grades (*i.e.* more marbling) receive a premium while lower grades receive a price reduction when marketed on a grid system (DiCostanzo and Dahlen 2000). Therefore feedlots have an economic incentive to maximize marbling in their cattle.

Vitamin A is thought to affect marbling by increasing the number of mature fat cells (adipocytes) within the muscle. Metabolites of vitamin A bind to nuclear receptors that then up or down regulate genes involved in adipogenesis, the formation of new fat cells (Ziouzenkova *et al.* 2007). Polymorphisms within the genes involved in this pathway could conceivably alter the impact of vitamin A on marbling.

The goal of this research was to discover a genetic marker that, in combination with manipulation of vitamin A supplementation, could be implemented as a marker-assisted management program to maximize marbling in beef cattle.

2 LITERATURE REVIEW

2.1 Vitamin A

2.1.1 Carotenoids and Retinoids

Carotenoids are plant-based yellow to red pigments. They are 40-carbon molecules that contain a polyisoprenoid structure, a chain of conjugated double bonds (Britton 1995). Greater than 600 different carotenoids have been characterized, but only approximately 10% can be metabolized to vitamin A (Yeum and Russell 2002). Carotenoids that can be converted to vitamin A are also known as pro-vitamin A. They are characterized by a β-ione ring and include such compounds as α-, β-, and ν-carotene and β-cryptoxanthin (NRC 1987). The relative biological vitamin A activity of pro-vitamin A varies between molecules. For example, β-carotene has the highest activity at 50% of all-trans-retinol (NRC 1987). Carotenoids are often tightly bound to macromolecules, which reduce their availability and subsequent biological activity (Parker 1996).

Retinoids include vitamin A and its related metabolites. There are four distinct forms of retinoid found within the body: retinyl esters (retinoid esterified to a long-chain fatty acid), retinol (vitamin A), retinaldehyde, and retinoic acid (Bonet et al. 2003; Figure 2.1). Several isomers of each retinoid exist, the most notable are all-*trans*, 9-*cis*, and 11-*cis*.

Retinyl esters of long-chain fatty acids are the storage form of retinoid. They are located primarily in the liver, but can also be found in adipose tissue (Blomhoff et al. 1982, Tsutsumi et al. 1992). Retinyl esters are formed from the esterification of retinol and fatty acids by lecithin:retinol acyltransferase, and they can be hydrolyzed by a variety of enzymes, including pancreatic triglyceride lipase and phospholipase B (Rigtrup et al. 1994, van Bennekum et al. 2000, Harrison 2005).

Retinol is the only form of retinoid that is considered true vitamin A. Other retinoids and pro-vitamin A carotenoids also have varying degrees of vitamin A activity, therefore three units have been developed to standardize the measurement of vitamin A activity in feedstuffs: international units (IU), retinol equivalents (RE), and retinol activity equivalents (REA). One IU is equivalent to 0.300 μg all-trans-retinol or 0.550 μg retinyl palmitate (NRC 1996). Both 1 RE and 1 REA are equal to 1 μg all-*trans*-retinol, however they differ with respect to the activity of carotenoids (FAO 1967; Institute of Medicine, Food and Nutrition Board 2001). International standards

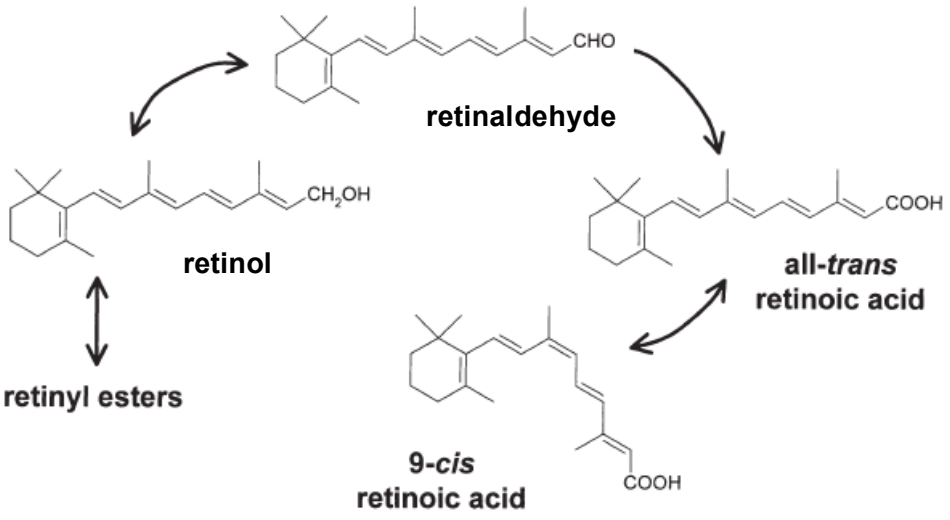

Figure 2.1 Diagrammatic representation of biologically significant retinoids. Modified from Bonet *et al.* 2003.

equate 1 mg β-carotene to 1,667 IU, however due to ruminal degradation that value is reduced to 400 IU for cattle (NRC 1970).

The first product of retinol oxidation is retinaldehyde (Duester *et al.* 1999). Several isomers of retinaldehyde are present within the body. The retina is a depot for 11-*cis*- and all-*trans*-retinaldehyde, where the isomerization between the two forms is an integral component of the visual pathway (Wald 1968). All-*trans*-retinaldehyde is also present in adipose tissue and appears to play a role in regulating adipogenesis (Ziouzenkova *et al.* 2007).

The second metabolite in the vitamin A pathway is retinoic acid, produced by the oxidation of retinaldehyde (Labrecque *et al.* 1995). As with retinaldehyde, multiple isomers of retinoic acid are present *in vivo*, most notably 9-*cis*- and all-*trans*-retinoic acid, which act as ligands for nuclear receptors (Heyman *et al*. 1992, Repa *et al* 1993).

2.1.2 Digestion and Absorption

Vitamin A is present in the diet as either retinoids from animal sources or pro-vitamin A carotenoids from plant sources (Harrison 2005). Due to their lipid-soluble and hydrophobic nature, carotenoids are incorporated into micelles in the small intestine. The capacity of a

micelle to take-up carotenoids is influenced by the fatty acid composition of the micelle as well as the polarity of the carotenoid (Yeum and Russell 2002). The greatest limiting factor of carotenoid digestion is their dissociation from the matrix of food particles (Parker 1996). The degree and rate of release depends on the nature of the matrix, particle size, and the efficiency of digestive enzymes.

Once incorporated into micelles, carotenoids are absorbed by the intestinal mucosa of the duodenum via passive diffusion (Parker 1996). Within the enterocytes, carotenoids are packaged into chylomicrons and released into the lymphatic system and subsequently into circulation (Yeum and Russell 2002). Once in circulation, the chylomicrons are degraded by lipoprotein lipase and the carotenoid-containing chylomicron remnants are absorbed by the liver.

Pro-vitamin A carotenoids can be hydrolyzed to either retinaldehyde or retinol. One molecule of β-carotene yields two molecules of retinaldehyde, whereas α-carotene and β-cryptoxanthin each yield one molecule of retinol (Harrison 2005). The central bond of β-carotene is cleaved by β-carotene 15,15'-oxygenase to form two molecules of retinaldehyde (Goodman and Olson 1969). β-carotene 15,15'-oxygenase is present in the cytosol of both enterocytes and hepatocytes, therefore cleavage can occur before or after transport from the intestine to the liver (Yeum and Russell 2002).

The predominant form of retinoid present in feedstuffs is retinyl esters of long-chain fatty acids (Plack 1965). Retinol must be hydrolyzed from the ester linkage before it can be absorbed. Pancreatic triglyceride lipase catalyzes this reaction in the lumen of the small intestine (van Bennekum *et al.* 2000). Additionally, the membrane-bound enzyme phospholipase B also hydrolyzes retinol from retinyl esters at the brush border (Rigtrup *et al.* 1994).

Retinol is absorbed by enterocytes through both passive diffusion (non-saturable) and carrier-mediated facilitated diffusion (saturable), though the specific transport protein involved has yet to be identified (Quick and Ong 1990, Harrison 2005). Once inside the enterocyte, retinol is rapidly bound by retinol-binding protein 2 (RBP2; Harrison 2005). RBP2 sequesters retinol, protecting it from enzymatic and non-enzymatic degradation as well as making it soluble within the aqueous environment of the cytosol (Napoli 1993). Retinol is then re-esterified with long-chain fatty acids (primarily palmitate and stearate) by lecithin:retinol acyltransferase (MacDonald and Ong 1988, Harrison 2005).

The retinyl esters are packaged into chylomicrons, which are then secreted into the lymphatic system (Harrison 2005). The chylomicrons enter circulation and are converted to chylomicron remnants by the peripheral tissues. While some extra-hepatic tissues (particularly bone marrow and the spleen) have a limited capacity to take up retinyl esters directly from chylomicrons and chylomicron remnants, the majority of retinyl esters in the chylomicrons are absorbed by the liver (Blomhoff *et al.* 1990). The chylomicron remnants are taken up by the liver, where the retinyl esters are hydrolyzed and re-esterified for storage (Blomhoff *et al.* 1982, Harrison 2005).

2.1.3 Storage and Transport

Hepatocytes are responsible for the uptake of retinyl esters from chylomicron remnants in the liver (Blomhoff *et al*. 1982). The retinyl esters are hydrolyzed to retinol by retinyl ester hydrolase as they enter the cell (Harrison and Gad 1989). The retinol is transported to the endoplasmic reticulum where it is then bound to retinol binding protein 4 (RBP4; Blomhoff *et al.* 1985, Blomhoff *et al.* 1990). The retinol-RBP4 complex is secreted from the hepatocytes, where it is then transferred to stellate cells (Blomhoff *et al*. 1982, Rask *et al*. 1983)

Hepatic stellate cells store approximately 80% of total body vitamin A (Senoo *et al*. 2007). Within stellate cells, vitamin A is stored as retinyl palmitate located in lipid droplets in the cytoplasm. The size and number of the droplets is dependent on the amount of retinyl ester stored (Wake 1980). When retinol is required it is mobilized directly from stellate cell stores and bound by RBP4 for transport in the plasma (Anderson *et al*. 1992).

RBP4 transports retinol in the plasma. It allows the hydrophobic retinol to be soluble in the aqueous environment of the blood and protects it from oxidation (Raghu and Sivakumar 2004). One mole of RBP4 transports one mole of all-*trans*-retinol. The retinol-RBP4 complex is bound to transthyretin in circulation, which prevents the low-molecular weight RBP4 (21 kDa) from being filtered through the kidneys (Vahlquist *et al*. 1973). The trans-membrane protein STRA6 (stimulated by retinoic acid 6) transports retinol from the RBP4 complex and into the cell (Wolf 2007). Retinol can also spontaneously diffuse through the plasma membrane without the aid of a transport protein (Fes and Johannesson 1987). Once the retinol has been delivered to the target cell, RBP4 dissociates from transthyretin and is then catabolized by the kidney (Goodman 1984, Berni *et al.* 1993). Within the cytosol, additional retinol binding proteins (RBP1, 2 and 5) sequester and protect retinol and its first metabolite retinaldehyde. Additionally, two classes of

cellular retinoic acid binding protein, CRABP1 and CRABP2, bind and protect retinoic acid (Napoli 1993).

Hepatic stellate cells regulate vitamin A homeostasis (Senoo *et al*. 2007). The cells have membrane-bound RBP4 receptors, which can take up RBP4-bound retinol from circulation if plasma retinol concentrations become too high and store the retinol as retinyl palmitate (Smeland *et al*. 1995, Senoo *et al*. 2007). If plasma retinol concentrations become too low, stellate cells will hydrolyze stored retinyl palmitate to retinol and secrete it into the bloodstream as a complex with RBP4 (Anderson *et al*. 1992, Senoo *et al*. 2007). Through these mechanisms plasma retinol concentration is tightly controlled.

2.1.4 Metabolism

Retinol is oxidized to retinaldehyde within the cytosol by alcohol dehydrogenase (ADH; Figure 2.2). ADH belongs to a larger family of medium-chain dehydrogenases/reductases. ADH catalyzes the oxidization of a broad range of substrates, including retinol and ethanol (Duester *et al.* 1999). ADH1 is the primary enzyme responsible for the metabolism of retinol to retinaldehyde in humans and rats (Molotkov *et al.* 2002a). It can also function in the reverse direction, reducing retinaldehyde to retinol (Boleda *et al.* 1993). Several other enzymes, including ADH3, ADH4, and short-chain dehydrogenases are also capable of this conversion but their activities are significantly lower than ADH1 (Duester 2000, Molotkov *et al*. 2002a). Retinaldehyde can be further oxidized to retinoic acid by aldehyde dehydrogenase (ALDH). ALDH is a super family of diverse enzymes that catalyze the oxidation of a wide range of endogenous and exogenous aldehyde substrates (Sophos and Vasiliou 2003). Aldehyde dehydrogenase 1A1 (ALDH1A1) is able to oxidize 9-*cis*-retinaldehyde to 9-*cis*-retinoic acid and all-*trans*-retinaldehyde to all-*trans*-retinoic acid with nearly equal efficiency (Labrecque *et al.* 1995, Duester 2000). It is the primary enzyme responsible for the metabolism of retinaldehyde to retinoic acid, as *ALDH1A1* knockout mice displayed a 77% reduction in retinoic acid synthesis following administration of retinol (Molotkov and Duester 2003).

Cellular retinoic acid concentrations are controlled in part by the CYP26 family of cytochrome P450 enzymes, which are activated via negative feedback from retinoic acid (White *et al.* 1997, Taimi *et al.* 2004). These enzymes oxidize retinoic acid to polar metabolites

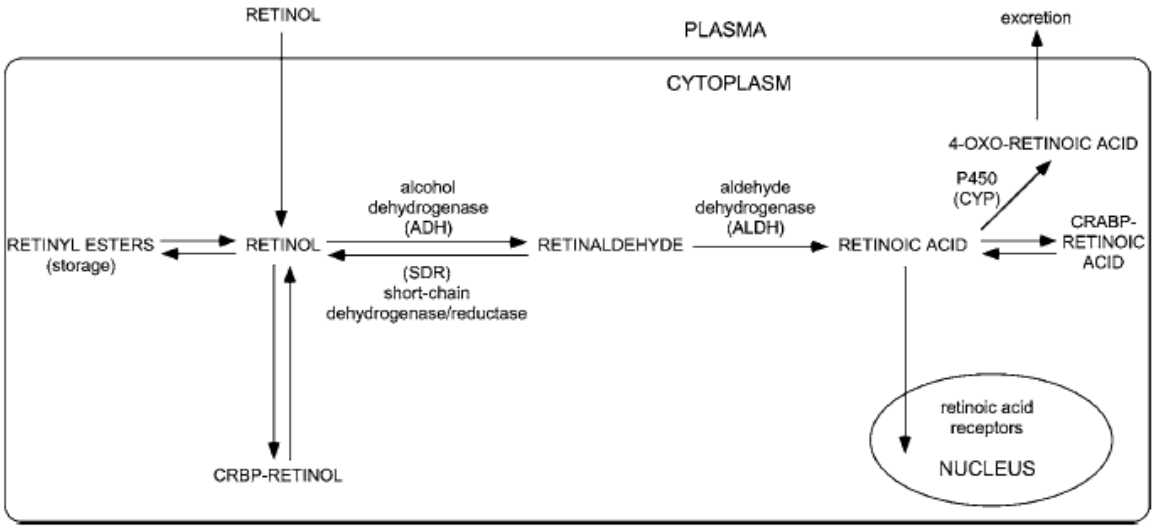

Figure 2.2 Schematic of retinol metabolism within the cell (Molotkov *et al*. 2004, with permission). Within the cytoplasm retinol can be bound to cellular retinol binding protein (CRBP), converted to retinyl esters for storage, or metabolized to retinaldehyde by alcohol dehydrogenase. Retinaldehyde can be reduced to retinol by short-chain dehydrogenase-reductase or oxidized to retinoic acid by aldehyde dehydrogenase. Retinoic acid is bound by cellular retinoic acid binding protein (CRABP) and transported to the nucleus (where it binds to retinoic acid receptors) or oxidized by cytochrome P450 (CYP) to 4-oxo-retinoic acid and excreted.

(including 4-*oxo*-, 4-*hydroxy*-, and 18-*hydroxy*-retinoic acid), which are then eliminated (Leo *et al.* 1984, White *et al*. 2000).

2.1.5 Functions

2.1.5.1 Vision

There are two types of light-sensing cells within the eye: rods and cones (reviewed by Wolf 2004). Cones perceive color in bright light, whereas rods function as black and white in dim light. The visual pathway of rod cells was first described by Wald (1968). In rod cells, 11-*cis*-retinaldehyde is bound to rhodopsin. When exposed to a photon of light the 11-*cis*-retinaldehyde is isomerized to all-*trans*-retinaldehyde. The rhodopsin is released and generates a neural signal. The all-*trans*-retinaldehyde is reduced to all-*trans*-retinol, which is then esterified to a long-chain fatty acid by lecithin:retinol acyltransferase. The retinyl ester is simultaneously hydrolyzed and

isomerized to 11-*cis*-retinol by isomerhydrolase. Alcohol dehydrogenase oxidizes the 11-*cis*-retinol to 11-*cis*-retinaldehyde, which is then bound by rhodopsin, completing the cycle.

Mata *et al*. (2002) elucidated a different visual pathway in cone cells. In cones, 11-*cis*-retinaldehyde is bound to opsin. Like in rod cells, 11-*cis*-retinaldehyde is isomerized by light to all-*trans*-retinaldehyde and releases its protein (which creates a neural impulse) and is then reduced to all-*trans*-retinol. However, unlike rod cells, in cone cells the all-*trans*-retinol is isomerized to 11-*cis*-retinol by isomerase and then esterified with palmitoyl CoA by estersynthase to 11-*cis*-retinyl palmitate. The 11-*cis*-retinyl palmitate is hydrolyzed to 11-*cis*-retinol and then oxidized to 11-*cis*-retinaldehyde.

2.1.5.2 Reproduction

Vitamin A is essential for the maintenance of pregnancy and fetal development; deficiency results in abortions, stillbirths, and weak offspring (Thompson *et al*. 1964). Retinol stimulates steroidogenesis in the ovaries, which impacts the uterine environment via the action of progesterone (Chew 1993). Vitamin A deficiency disrupts the estrus cycle and results in reduced conception rates (Bates 1983).

Retinol is necessary for spermatogenesis and maintenance of the germinal epithelium in the testes (Appling and Chytil 1981, Chew 1993). Retinol deficiency results in testicular atrophy and the cessation of sperm production. Additionally, retinoic acid is required for testosterone synthesis (Appling and Chytil 1981).

2.1.5.3 Immunity

Vitamin A deficiency has been associated with increased morbidity and mortality of many infectious diseases, including diarrhea, respiratory infections, leprosy, and measles (reviewed by Semba 1994). Deficiency causes keratinization of mucous membranes as well as a reduction in goblet cells and decreased mucous production. Deficiency-induced deterioration of the spleen and thymus results in changes in T-cell subpopulations (Blackfan and Wolbach 1933). Retinoic acid enhances the proliferation of thymocytes and tonsillar lymphocytes in response to mitogens and stimulates the transcription of IL-2 and IL-2 receptors in T lymphoblasts (Sidell *et al*. 1981,

Sidell *et al*. 1984). Vitamin A deficiency results in decreased production of retinoic acid, thereby impeding immune response.

2.1.5.4 Gene Expression

Retinoic acid regulates gene expression by binding to nuclear receptor proteins, which then form complexes and bind to nuclear response elements (Figure 2.3). Both 9- *cis*-retinoic acid and all-*trans*-retinoic acid are ligands for retinoic acid receptors (RAR). In contrast, only 9-*cis*-retinoic acid is a ligand for retinoid X receptors (RXR; Heyman *et al*. 1992, Repa *et al* 1993). Retinaldehyde, the precursor of retinoic acid, inhibits the action of retinoic acid, possibly by competing with retinoic acid as a substrate for RXR and RAR.

There are three types each of RXR and RAR, α, β, and γeach with multiple isoforms. The types and isoforms differ with respect to their tissue and temporal patterns of expression (Chambon 1996). RXR can form homodimers with itself or heterodimers with a variety of other nuclear receptor proteins including RAR, peroxisome proliferator-activated receptors (PPARα, β, and γ), vitamin D receptors, thyroid hormone receptors, and liver X receptors (Bugge *et al*. 1992, Zhang *et al*. 1992, Bardot *et al*. 1993, DiRenzo *et al*. 1997, Desvergne 2007). The homo and heterodimers, when bound to their respective ligands, bind to retinoic acid response elements (RARE) located within the promoter region and act to up-regulate or down-regulate expression of particular genes (Figure 2.3; Heyman *et al*. 1992, Ziouzenkova *et al.* 2007). The RARE motif is generally characterized as two repetitions of the hexameric motif 5'-PuG(G/T)TCA separated by one to five base pairs (Chambon 1996). RXR heterodimers can also bind to the response elements of the other partner, such as PPARγ/RXR to a PPAR-responsive element (Iwaki *et al.* 2003).

2.1.6 Cattle

2.1.6.1 Requirements

The primary sources of pro-vitamin A carotenoids in cattle diets are high quality forage and silage (NRC 1996). The carotenoid content of hay is highly variable and decreases dramatically with time (Guilbert 1935, Cullison and Ward 1965). In general, forages are good sources of vitamin A where as grains typically contain little vitamin A (Table 2.1). The National Research

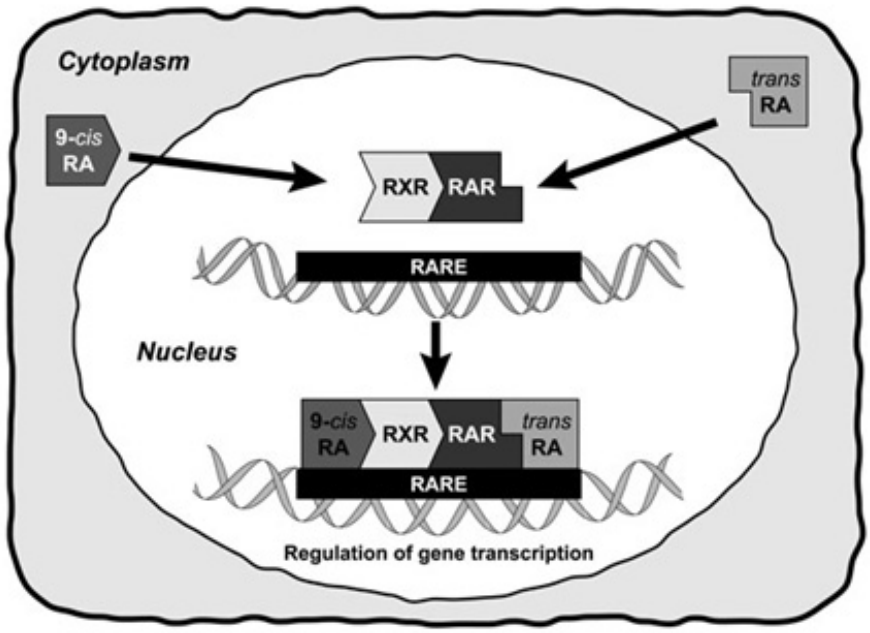

Figure 2.3 Model of retinoic acid (RA) regulation of gene expression (Higdon 2011, with permission). In the nucleus 9-*cis*-retinoic acid binds to a retinoid X receptor (RXR) and all-*trans*-retinoic acid binds to a retinoic acid receptor (RAR) of a RXR/RAR heterodimer. This complex then binds to the retinoic acid response element (RARE) of a chromosome and acts to regulate transcription.

Table 2.1 The vitamin A content of common feed ingredients in beef cattle diets (NRC 1996).

Feed Stuff	Vitamin A (IU/kg dry matter)
Alfalfa hay (mid bloom)	46,000
Alfalfa silage	155,000
Barley grain	3,800
Corn	1,000
Dried distillers grains with solubles	1,200
Spring pasture	184,000

Council reports the minimum vitamin A requirement for feedlot cattle as 2,200 IU/kg dry matter, 2,800 IU/kg for pregnant heifers and cows, and 3,900 IU/kg for lactating cows and breeding

bulls (NRC 1996). The presumed upper safe level of vitamin A for all types of cattle is 66,000 IU/kg dry matter (NRC 1987).

The most characteristic symptoms of vitamin A deficiency are those that involve the eye. Night blindness (nyctalopia) is the only condition that is unique to vitamin A deficiency (NRC 1987). Other ocular symptoms include lacrimation (excessive tear production), xeropthalmia (dry eyes and corneal thickening), corneal ulceration, and corneal keratinization (NRC 1987, West 1994). The milder conditions are reversible with vitamin A supplementation, however corneal damage can cause permanent blindness (West 1994). Other symptoms associated with vitamin A deficiency include reduced feed intake, edema, impaired fertility, abortions/stillbirths, increased susceptibility to infection, and ultimately death (NRC 1987).

Animals have a high tolerance for vitamin A; therefore chronic toxicity requires long-term exposure to 100 to 1000 times the required intake (NRC 1987). Toxicity can either be acute or chronic. Acute toxicity is apparent within hours of a massive dose. Symptoms include loss of appetite, nausea, peeling skin, muscle weakness, twitching, convulsions, and death (NRC 1987). The most notable indications of chronic toxicity are skeletal malformations and internal bleeding. Other symptoms include reduced growth, thickening of the skin, reduced blood clotting, anemia, and conjunctivitis (NRC 1987). Vitamin A is also a potent teratogen, therefore care must be taken to prevent over exposure during pregnancy (Nau *et al*. 1994).

2.1.6.2 Restriction to Improve Carcass Traits

Several feeding studies involving vitamin A restriction or supplementation have been performed on feedlot cattle. In general, vitamin A level did not have an effect on average daily gain, dry matter intake or feed to gain ratio (Gorocica-Buenfil *et al.* 2007a, Gorocica-Buenfil *et al*. 2007b, Cusack *et al*. 2008, Gorocica-Buenfil *et al.* 2008), however Oka *et al*. (2004) did find that steers injected with supplemental vitamin A had greater average daily gain, carcass weight, and subcutaneous fat than non-supplemented steers. Vitamin A restriction is associated with increased marbling and intramuscular lipid concentration (Oka *et al.* 1998, Oka *et al*. 2004, Gorocica-Buenfil *et al*. 2007a, Gorocica-Buenfil *et al*. 2007b, Wang *et al.* 2007, Siebert *et al*. 2006). Consequently, serum retinol concentration is negatively correlated to marbling (Torii *et al*. 1996, Oka *et al*. 1998). Vitamin A restriction also resulted in an increase in the mono-

unsaturated fatty acid content of subcutaneous adipose tissue (Siebert *et al*. 2006, Gorocica-Buenfil *et al.* 2008).

2.2 Fat Deposition

2.2.1 Growth and Development

At birth cattle possess two distinct forms of adipose tissue: white and brown (Alexander *et al.* 1975). Brown adipose tissue is characterized by multilocular lipid distribution (many small droplets) and large number of mitochondria within the adipocytes (Heaton 1972). Its primary purpose is for nonshivering thermogenesis, which is critical for the thermoregulation of newborn animals (Dawkins and Hull 1963). At birth, all but the subcutaneous depot of the calf are brown adipose tissue, but by 30 days of age all of the fat depots change to almost entirely white adipocytes (Alexander *et al*. 1975).

White adipocytes are unilocular (one large lipid droplet) and range in average diameter from 73 to 183 μm in cattle (Cianzio *et al*. 1985). The most notable function of white adipose tissue is the storage of energy in the form of triglycerides. It is also an endocrine organ, secreting many products including leptin, resistin, and angiotensinogen (Zhang *et al.* 1994, Safonova *et al.* 1997, Steppan *et al*. 2001, reviewed by Trayhurn and Beattie 2001).

The formation and growth of adipose tissue involves both hyperplasia (increased cell number) and hypertrophy (increased cell size). Adipogenesis is the process of proliferation of preadipocytes and their differentiation into mature adipocytes. Hypertrophy of adipocytes occurs as they accumulate triglycerides created through lipogenesis (reviewed by Hausman *et al*. 2001).

Total body fat content of cattle increases with age. In crossbred beef steers total dissectible fat (subcutaneous, intermuscular, mesenteric, kidney, and brisket fat) increased from 22.5% of carcass weight at 11 months of age to 42.9% at 19 months of age (Cianzio *et al*. 1985). Intermuscular fat was the largest depot in all ages studied and the amount of subcutaneous fat remained approximately 60% that of intermuscular fat. Intramuscular and brisket fat were the latest depots to develop (Cianzio *et al*. 1985)

The average diameter of adipocytes in beef steers increased from 11 to 17 months but did not change from 17 to 19 months, indicating that adipose depots grew first primarily through hypertrophy (Cianzio *et al*. 1985). Hyperplasia was observed in intramuscular and brisket fat but not in subcutaneous or intermuscular fat. Hyperplasia is the primary mechanism of

intramuscular adipose growth as cell number but not cell size is positively correlated with percentage intramuscular lipid (Hood and Allen 1973).

The pattern of fat deposition differs with type of cattle. At 14 months of age Hereford x Angus cross steers had significantly greater subcutaneous fat (both thickness and weight) than Holstein steers of the same age and weight (Hood and Allen 1973). This depot was due to both greater cell number and cell diameter. No difference was found in fat as a percentage of carcass weight between age-matched large and small frame crossbred beef steers slaughtered between 11 to 19 months of age (Cianzio *et al*. 1982). Additionally, no difference between frame sizes was found for each fat depot (subcutaneous, intermuscular, kidney, pelvic, omental, mesenteric) as a percentage of total body fat. However, when each depot was adjusted for total body fat mass, large frame steers had significantly greater kidney and less subcutaneous fat than small frame steers. As well, the growth rates of pelvic and mesenteric fat depots decreased with age in the large frame steers but did not change in the small frame steers, indicating that the pattern of fat deposition differs with frame size in crossbred beef steers.

2.2.2 Adipogenesis

The first stage of adipogenesis is proliferation of preadipocytes. Proliferation is controlled by hormonal and neural factors (Hausman *et al*. 2001). Insulin-like growth factor 1 (IGF-1), produced by mature adipocytes, strongly stimulates and is necessary for proliferation (Hausman *et al.* 1993, Hausman *et al*. 2001). Adipose tissue is innervated by the sympathetic nervous system, which has an inhibitory effect on proliferation (Cousin *et al*. 1993).

Following proliferation, pre-adipocytes enter growth arrest and terminally differentiate into mature adipocytes capable of lipid accumulation (Scott *et al*. 1982, Negrel 1994, Hausman *et al.* 2001). Adipocytes are considered to be terminally differentiated when they express cellular and/or molecular markers that indicate they are capable of assimilating fatty acids into triglycerides (Hausman *et al*. 2009).

IGF-1, in addition to stimulating proliferation, is also essential for differentiation (Smith *et al*. 1988). Growth hormone stimulates differentiation by increasing IGF-1 production by preadipocytes (Gaskins *et al.* 1990, Cornelius *et al*. 1994). Growth hormone also has an antagonistic effect on adipogenesis as it promotes lipolysis and therefore causes a reduction in mass of adipose tissue depots (reviewed by Hausman *et al*. 2009).

The CCAAT/enhancer binding protein (C/EBP) family of transcription factors are crucial for differentiation of preadipocytes into mature adipocytes. Production of C/EBPβ and C/EBPδ increases dramatically in the early stages of differentiation, peaking at days two to four of cell culture (Yeh *et al*. 1995, Rosen *et al*. 2000). As levels of C/EBPβ and C/EBPδ decrease, C/EBPα is produced, peaking at days six to eight, by which time the adipocytes appear morphologically differentiated (Yeh *et al*. 1995).

Several genes involved in controlling adipogenesis are influenced by retinoic acid and retinaldehyde. As many as 11 different RXR heterodimers are involved in the regulation of adipogenesis (Fu *et al.* 2005, Ziouzenkova *et al.* 2007). RXRα is involved in adipocyte differentiation via heterodimer formation with peroxisome proliferator-activated receptor γ (PPARγ by binding to a PPARγ-responsive element located in the promoter region of the gene *adiponectin* (Imia *et al*. 2001, Iwaki *et al*. 2003). Transgenic mice lacking expression of RXRα in adipocytes were resistant to diet-induced obesity. The same mice also displayed impaired lipolysis in response to fasting, indicating that RXRα (and therefore its ligand 9-*cis*-retinoic acid) is essential for fatty acid liberation from adipocytes (Imia *et al*. 2001).

Retinaldehyde inhibits adipogenesis *in vivo* and *in vitro*. *ALDH1A1* knockout mice were found to be resistant to diet-induced obesity and subsequently displayed a significant reduction in adipogenesis and a higher metabolic rate (Ziouzenkova *et al*. 2007). In the same study, obese (*ob/ob*) mice administered retinaldehyde or an ALDH1A1 inhibitor displayed a decrease in subcutaneous and visceral fat. Administration of vitamin A (all-*trans*-retinol) or all-*trans*-retinoic acid did not have a significant effect. While retinaldehyde does not directly activate RXR it has been found to significantly inhibit 9-*cis*-retinoic acid activation of RXRα (Ziouzenkova *et al*. 2007). It may be by inhibiting RXR-mediated activation of adipogenic genes that retinaldehyde inhibits adipogenesis.

2.3 Genetic Polymorphisms

2.3.1 Types of Single Nucleotide Polymorphisms

A single nucleotide polymorphism (SNP) is a single nucleotide change between individuals at a specific locus within the genome (Lewin 2008a). SNPs are abundant in the genome; it is estimated that in the human genome approximately one SNP occurs every 1300 bp (Altshuler *et*

al. 2000, Lewin 2008a). There are over 4.9 million *Bos taurus* SNP submissions in the current build (build 131) of dbSNP (the National Center for Biotechnology Information SNP database), and at least 2.4 million additional submissions to be included in the next build (NCBI 2010).

SNPs can either be coding or non-coding, and coding SNPs can be further categorized as nonsense, missense, or silent. Nonsense SNPs introduce a premature stop codon into the transcribed mRNA, resulting in a truncated protein. The truncated protein is often non-functional and rapidly degraded (reviewed by Hentze and Kulozik 1999).

Missense SNPs result in an amino acid substitution within the protein. Non-conserved mutations (which change the class of amino acid) and the addition or removal of cysteine are generally more severe than conserved mutations (which change the amino acid to one within the same class) because they can impact the folding and subsequently the functioning of the protein (Pey *et al*. 2007). Conserved mutations can impact protein function if they occur within a critical motif (Honkatukia *et al*. 2005).

A silent mutation is a SNP within the coding region that does not result in the change of an amino acid. Though they do not alter the resultant protein, recent studies have revealed several mechanisms by which silent mutations can have a profound biological effect. If silent SNPs occur within exonic splicing enhancers or splice sites (the first and last two bp of the exon) they can interfere with splicing and result in aberrant mRNAs (reviewed by Cartegni *et al*. 2002). Silent SNPs can also alter mRNA folding and stability, both of which can reduce protein translation (Duan *et al.* 2003, Kudla *et al*. 2009). Lastly, a substitution of a rare codon can cause a pause in translation, which has been shown to cause protein misfolding (Kimchi-Sarfaty *et al.* 2007).

SNPs within the non-coding regions of a gene can also have significant biological effects. A SNP within a splice site, highly conserved regions located at the 5' and 3' ends of introns, or within the branch site can impede splicing and result in exon skipping (Lewin 2008b). The promoter region, approximately 200 to 2000 bp in length, contains binding sites for the basal transcription apparatus and transcription factors (Blanco *et al.* 2006). SNPs in this region could add or remove binding sites, which could significantly alter expression of the gene (Napierala *et al*. 2005). Lastly, SNPs in the 3' untranslated region (UTR) can alter mRNA stability, thereby changing the steady-state level of expression (Chen *et al.* 2006)

2.3.2 Candidate Genes

2.3.2.1 *Alcohol Dehydrogenase 1C (ADH1C)*

ADH1 is the primary enzyme responsible for the oxidation of retinol to retinaldehyde (Molotkov *et al.* 2002a). In humans and primates there are three distinct forms, *ADH1A, ADH1B,* and *ADH1C*, which share approximately 94% amino acid and greater than 90% coding sequence identity (Duester *et al*. 1999, Duester 2000, Oota *et al*. 2007). *ADH1A* arose from a duplication of *ADH1C* that occurred approximately 54 to 84 million years ago, and *ADH1B* arose from a duplication of *ADH1A* approximately 44 to 70 million years ago (Oota *et al*. 2007). ADH1C has the highest affinity for retinol, nearly 10 fold higher than that of ADH1A or ADH1B (Höög *et al*. 2001).

There is only one known form of *ADH1* in rodents (Molotkov *et al*. 2002b), and it is unclear whether one or multiple forms are present in cattle. Full coding sequence is available for cattle *ADH1C* (GenBank NM_001206387). The gene contains nine exons which result in a 1327 bp mRNA that codes for a 375 amino acid protein. Expressed sequence tags indicate that it is expressed in the liver, kidneys, skin, and testes, but not in adipose tissue (UniGene Bt.23740).

An entry for cattle *ADH1A* on BTA 6 has been made in GenBank (GeneID 280982), though no sequence information is available. A BLAST search of human *ADH1A* exons against the *Bos taurus* reference sequence did not produce any other *ADH1* forms.

2.3.2.2 *Aldehyde Dehydrogenase 1A1 (ALDH1A1)*

The aldehyde dehydrogenase superfamily encompasses a diverse array of aldehyde metabolizing enzymes. There are twenty known families of aldehyde dehydrogenases in eukaryotes (Sophos and Vasiliou 2003). Aldehyde dehydrogenase 1A1 (ALDH1A1), a member of family 1, catalyzes the oxidation of retinaldehyde to retinoic acid (Labrecque *et al.* 1995). *ALDH1A1* is located on BTA 8 and contains 13 exons which produce a 2071 bp mRNA (GenBank NM_174239). It is expressed in many tissues, including adipose and liver (UniGene Bt.4732).

2.3.2.3 *Retinol Binding Protein 4 (RBP4)*

RBP4 is the sole protein responsible for the transport of retinol from the liver to other tissues (Berni *et al*. 1993). In humans, serum RBP4 level is correlated with insulin resistance, intramuscular lipid concentration, intrahepatic lipid concentration, and abdominal fat mass (Perseghin *et al*. 2007, Högström *et al*. 2008). *RBP4* is located on BTA 26 (GenBank NM_001040475). The gene contains four exons and codes for a 201 amino acid protein.

RBP4 is produced by hepatocytes and is released into circulation bound to one molecule of all-*trans*-retinol (Soprano and Blaner 1994, Quadro *et al*. 2003). In the plasma it is complexed with transthyrine (TTR) in a 1:1 ratio (Vahlquist *et al*. 1973). It is thought that the purpose of this complex is to prevent filtration of RBP4 through the kidneys. Once the retinol has been delivered to the target cell RBP4 dissociates from TTR and due to its small size is catabolized by the kidneys (Goodman 1984, Berni *et al.* 1993).

2.3.2.4 *Peroxisome Proliferator-Activated Receptor γ (PPARγ)*

PPARγ is located on BTA 22 and contains 7 exons that result in an 1815 bp mRNA (GenBank NM_181024). Two isoforms of *PPARγ* (*PPARγ1* and *PPARγ2*) are expressed by bovine adipocytes, however only PPARγ2 has been shown to participate in adipocyte differentiation (Sundvold *et al*. 1997, Ren *et al.* 2002).

PPARγ is integral to the differentiation of adipocytes during adipogenesis. Expression of PPARγ2 is sufficient to induce differentiation of preadipocyte cell cultures (Tontonoz *et al*. 1994a). It forms a heterodimer with RXR, which then activates the transcription of adipogenic genes such as *fatty acid binding protein 4* (Kliewer *et al.* 1992, Tontonoz *et al*. 1994β). Adipose hypertrophy in response to vitamin A restriction has been associated with increased PPARγ2 expression in rats (Ribot *et al*. 2001). It is likely that a similar response could be observed in cattle following vitamin A restriction.

3 HYPOTHESES

A significant amount of research indicates that vitamin A restriction is associated with increased intramuscular fat deposition in cattle, but the mechanism remains unclear. Vitamin A exerts its effects through the binding of retinoic acid to nuclear receptors, which in turn up or down regulate the expression of target genes. The increased marbling observed in cattle is likely the result of nutritional and genetic interactions; *i.e.* nutrigenetics.

We hypothesize that polymorphism(s) in genes in the vitamin A metabolism pathway (*ADH1C*, *ALDH1A1*, *PPARγ*, and *RBP4*) in cattle will reduce metabolism of retinol to retinaldehyde and retinoic acid. Under normal feeding conditions, where vitamin A is plentiful, sufficient retinaldehyde and retinoic acid will be produced and therefore no difference will be observed between different genotypes of cattle. When vitamin A is limited there will be genotypic differences in the conversion of retinol to retinaldehyde and retinoic acid, resulting in phenotypic differences in intramuscular fat deposition.

4 MATERIALS AND METHODS

4.1 SNP Discovery

The candidate genes *ADH1C*, *PPARγ*, *ALDH1A1*, and *RBP4* were evaluated for SNPs. The mRNA sequence for each gene (GenBank NM_001206387, NM_181024, NM_174239, and NM_001040475) was aligned with the *Bos taurus* reference genome using Sequencher 4.9 software (Genecodes, Ann Arbor, MI). Primers were designed using Primer3 software (Rozen and Skaletsky 2000). For *ADH1C* (9 exons), *PPARγ* (7 exons), and *RBP4* (4 exons), the primers were designed to flank each exon, as well as portions of the 5' UTR and 3'UTR, from genomic DNA (Table 4.1). Due to the large size of *ALDH1A1* (13 exons) four sets of overlapping primers were designed to amplify the coding sequence from fat and muscle cDNA. Additionally, primers were designed to amplify *ALDH1A1* exons 3, and 13, and portions of the 5'UTR and 3' UTR from genomic DNA. The primers were obtained from Operon Biotechnologies Inc. (Huntsville, AL) and Integrated DNA Technologies (Coralville, IA).

Each fragment was amplified by PCR from genomic DNA from fourteen parents of the Canadian Beef Reference Herd (three Charolais, three Hereford, three Simmental, two Angus, two Limousin, and one Belgian Blue; Schmutz *et al*. 2001) or from fat and/or muscle cDNA from seven of their progeny. Each 20 µl PCR cocktail consisted of 0.2 pmol forward and reverse primers, 0.2 mM dNTPs (Fermentas, Burlington, ON), 10% Jeffrey's buffer (45 mM Tris HCl, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7 µM β-2-mercaptoethanol, 0.45 mM EDTA, and 0.25 mM spermidine), and 0.5 units Taq polymerase (Fermentas). The PCR program, performed on a Robocycler Gradient 96 thermocycler (Stratagene, La Jolla, CA), began with an initial denaturation at 94˚C for 4 minutes followed by 35 cycles of 1 minute at 94˚C, 50 seconds at the annealing temperature (Table 4.1) and 50 seconds at 72˚C. The program was concluded with a final extension period at 72˚C for 3 minutes.

The PCR products were electrophorized on a 1% agarose gel. The bands were excised and the DNA was extracted using a QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON). The fragments were sequenced in the forward and reverse directions by the Plant Biotechnology Institute (Saskatoon, SK). Sequences were aligned and analyzed with Sequencher version 4.9 software (Genecodes, Ann Arbor, MI). P-Match software (Chekmenev *et al*. 2005) was used to

Table 4.1 Primers used to amplify segments of *Bos taurus ADH1C*, *PPARγ*, *ALDH1A1*, and *RBP4* genes for sequencing.

Target	Forward Primer	Reverse Primer	Annealing (˚C)	Size (bp)
ADH1C				
5' UTR	TGCTGCTTTCTTACTCCTCCTTGC	CAGGCTCTTCCCTGTTCAAG	62	388
Exon 1	CAGGGCTTAAAGATCCCAGA	TAGCCAATGCTTGTCTCTCG	63	250
Exon 2	GGGGACAATGGTGTCATCTC	CAGCTTCCCTTGACAACAAA	58	267
Exon 3	TTCTTCAATGCAACTTCAGGA	GAGCCAAGGCACATTTCCTA	59	268
Exons 4-5	AAGAAGAATCTGTTTTATCCTCATCC	TGCTTTGCTTTTAGCTCGTG	62	498
Exon 6	GGCGGATTCTTTACCACTGA	TCTTTCAGCACCTCCTCGAT	60	380
Exon 7	CTCTTCCAGCCACACACTCA	TTCAGTCGTGTCGGACTCTG	62	357
Exon 8	CTCAGCAACAGTAGTCTGTGGA	GTCAAACCCCACCAAAACTT	66	290
Exon 9	GGAATGGGAATGGAATAGCA	CTTTTGGAAAGCTCCCATGT	55	291
3' UTR	AATTGCACATGGGAGCTTTC	AGGGTGGCAAAGACACAAAA	60	221
PPARγ				
5'UTR	TTGCCAAAGCAGTGAACAG	GGGATTTGCCACGACTCC	56	250
Exon 1	AAATATCGGTGGGAGTCGTG	CACTTGGAAGACAAGCCACA	65	210
Exon 2	CAGTCCTCAGGGCTAACGTC	AGTCTCAACAGTCTGAAAAGTGAAA	58	310
Exon 3	TTTTCCTGTGATGATTGTCTGC	TGCAACACGAAATGAAAACC	62	283
Exon 4	TTGACGGAACGTGTCAAGAG	CTGAAATCTGGCGATCTGTG	64	372

Target	Forward Primer	Reverse Primer	Annealing (˚C)	Size (bp)
PPARγ				
Exon 5	CTCATTCATCCTGCCCTTTC	CCACCCCAAATGAAGACAGT	63	264
Exon 6	TCAGCCACTTTCTCCCATTT	GAAAACCGCCAGCAGCAG	57	504
Exon 7	TTTGCCACTGCGTTCTGTCAACTG	ATGCTGTCAGTGAACTCGGACT	63	550
3'UTR	GTCCGAGTTCACTGACAGCA	GAAGGGAAGGGGAATGAAAG	56	285
ALDH1A1				
5'UTR	GGCTTTGTAAATTAATTCGTCTGC	GAGGACGACATTGCTGGTTC	66	300
cDNA[1] 1	CAGGAGCGGAACCAGCAATG	CCACACACACCAACAGGCT	58	508
cDNA 2	TATCTGATGGATTTAGGAGGCTGC	CGGCATCAGCAAACACAATG	61	493
cDNA 3	GACAGAGGTTGGCAAACTGAT	GCTGCACAGGTCCAAATATCTC	64	497
cDNA 4	CTCTGATGTTACTGATGATATGCGC	TTGGGTGTCATAGCAGAAGGC	64	548
Exon 3	GACACGACTGACGCGACTTA	TTTGAGTTCTGCCCAGTTCA	60	242
Exon 13 & 3'UTR	TCCCTAGCCAGATAAGCAGGAACA	TTGGGTGTCATAGCAGAAGGC	65	376
RBP4				
Exon 1	TCGCCTTGCTGGCTCCAC	AAAGGGCTTCCTCCCTCCAT	64	408
Exon 2	GCTCAGAATCCCCTTGGTTT	GGAGAAGAAACCCAGCCAGT	62	209
Exon 3	AGTCCCCGTGGCTTTCTG	GCAGATGTAACGCATCTCAGG	61	300
Exon 4	ACCAGTCCTAAGGCCATCCT	GAGGGGAGGTGAAGCAAACT	62	220

[1]*ALDH1A1* was sequenced from cDNA in four overlapping sections (cDNA 1-cDNA 4).

discover if SNPs found within the 5' UTR occur within potential binding motifs of transcription factors.

4.2 SNP Selection

A PCR-RFLP was designed to genotype each of five SNPs: *ADH1C c.-64T>C*, *ADH1C c.967C>T*, *PPARγ c.1334G>T*, *RADLH1 c.*27C>T*, and *RADLH1 c.*109A>G* (Table 4.2). PCR products were amplified (4 minutes at 94˚C, 35 cycles of 1 minute at 94˚C, 50 seconds at the annealing temperature and 50 seconds at 72˚C, followed by 3 minutes at 72˚C), digested with a restriction endonuclease (New England Biolabs, Ipswich, MA), and visualized via electrophoresis through an ethidium bromide-stained 2% agarose gel.

A population of 400 crossbred steers for which production and carcass data were available (Pugh 2007) were genotyped at each SNP for a preliminary search for associations between genotype and production or carcass characteristics. These steers were group-housed in pens and all fed the same standard barley-based backgrounding and finishing diets. SAS version 9.2 (SAS Institute Inc., Cary, NC) was used for statistical analysis and significance was set at $P<0.05$. Genotype at each SNP was analyzed as a one-way ANOVA (Proc Mixed) for association with production and carcass traits. Means were separated using Tukey's LSD.

To assess the allele frequency of *ADH1C c.-64T>C* in various breeds, 50 unrelated cattle from each of the following breeds were genotyped for the SNP: Angus, Charolais, Hereford, Limousin, and Simmental. A chi-square test was used to determine if the allele frequencies were significantly different between the breeds ($P<0.05$).

Table 4.2 PCR-RFLPs designed to genotype for five different SNPs. For each SNP the primers were used to amplify a PCR product that was then digested with a restriction enzyme to yield the reported fragment lengths.

SNP	Primers	Annealing (˚C)	Restriction Enzyme	Digest	Fragment Lengths (bp)
ADH1C c.-64T>C	CAGGGCTTAAAGATCCCAGA	63	*BslI*	5 hours, 55˚C	T = 250
	TAGCCAATGCTTGTCTCTCG				C = 160+93
ADH1C c.9687C>T	CTCAGCAACAGTAGTCTGTGGA	60	*CviKI-1*	4 hours, 37˚C	T = 170+51
	TTTCAAGTGGTAAAACATGGGTA				C = 123+51+47
PPARγ c.1344G>T	TTTGCCACTGCGTTCTGTCAACTG	65	*PstI*	6 hours, 37˚C	T = 550
	ATGCTGTCAGTGAACTCGGACT				G = 347+207
*ALDH1A1 c.*27C>T*	TCCCTAGCCAGATAAGCAGGAACA	65	*MnlI*	4 hours, 37˚C	T = 376
	TTGGGTGTCATAGCAGAAGGC				C =2 06+172
*ALDH1A1 c.*109A>G*	TCCCTAGCCAGATAAGCAGGAACA	65	*HpyCH4IV*	4 hours, 37˚C	A = 376
	TTGGGTGTCATAGCAGAAGGC				G = 281+93

4.3 Vitamin A Restriction Study

A nutrigenetic study was performed to investigate the effects of the interaction between *ADH1C c.-64T>C* genotype and vitamin A restriction on production and carcass traits in beef cattle. This project was approved by the University of Saskatchewan Animal Care Committee (protocol number 20090076) and was performed in accordance with the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care 1993).

4.3.1 Animals

In order to obtain animals of each genotype an initial population of 450 black Angus cross steers was purchased from a commercial auction market and housed at the University of Saskatchewan Beef Research Facility. Black Angus animals were chosen for this study because McLean and Schmutz (2009) found that cattle with at least one E^D allele at *melanocortin 1 receptor* (and therefore black in colour) had significantly greater backfat and average fat than cattle that were homozygous ee (and therefore red).

The animals were processed within 24 hours of arrival, at which time they were treated with Ivomec™ (Merial Canada Inc., Baie d'Urfe, QC) and long acting Liquamycin (Pfizer Animal Health, New York, NY) and vaccinated with Bovishield Gold 5 (Pfizer Animal Health) and UltraBac 7/Somnubac (Pfizer Animal Health). In order to maximize marbling potential, the steers did not receive hormonal implants, as anabolic implants have been associated with reduced intramuscular fat deposition in beef cattle (Duckett *et al*. 1999). A blood sample was also collected for DNA extraction (Montgomery and Sise 1990). Each animal was genotyped at *ADH1C c.-64T>C* (Table 4.2) and 130 steers were randomly selected by genotype (50 TT, 50 CT, and 30 CC) for use in the nutrigenetic study.

The steers (n=130) were group housed in four pens with an even distribution of genotypes between pens. They were backgrounded for three months on a vitamin A deficient diet (Table 4.3) to deplete their liver vitamin A stores. Following backgrounding, all of the steers were finished for five months on a barley grain and silage-based diet that did not contain any supplemental vitamin A (Table 4.3). The β-carotene content of each feed ingredient was analyzed by DSM Nutritional Products Inc. (Strathmore, AB; Appendix B).

Table 4.3 Composition and nutrient analysis of the backgrounding and finishing diets.

	Backgrounding	Finishing
Diet Composition (% DM[1])		
Oat Hulls	29.6	-
Barley Straw	14.6	-
Barley	29.6	81.7
Barley Silage	-	6.8
Wheat DDGS	21.1	5.8
Supplement	5.2	5.7
Supplement Composition (% DM)		
Barley	54.6	54.6
Limestone	26.1	26.1
Ionophore Premix[2]	9.6	9.6
Trace Mineral Salt[3]	6.5	6.5
Canola Oil	3.2	3.2
Nutrient Analysis		
Moisture (%)	6.6	7.2
Crude Protein (% DM)	11.6	13.3
Acid Detergent Fibre (% DM)	27.4	8.7
Neutral Detergent Fibre (% DM)	57.7	22.7
Ca (% DM)	0.5	0.6
P (% DM)	0.3	0.5
Vitamin A (IU/kg DM)[4]	22	549

[1]Dry matter.
[2]University of Saskatchewan Feed Unit ionophore premix. Contained (dry matter basis) 96.77% barley and 2.33% Rumensin© Premix (Elanco, Guelph, ON), containing 200 g/kg monensin as monensin sodium.
[3]Trace mineral salt. Contained 95% NaCl, 12,00 ppm Zn, 10,000 ppm Mn, 4,000 ppm Cu, 400 ppm I, 60 ppm Co, and 30 ppm Se.
[4]Calculated as 400 IU/mg β-carotene (NRC 1970).

The steers were randomly allocated (maintaining an even distribution between pens) to one of two vitamin A supplementation treatments: either unsupplemented (0 IU/month) or

supplemented (750,000 IU/month). The treatments were delivered as an oral bolus every four weeks for the duration of the finishing period (a total of five times). The boluses consisted of gelatin capsules (Torpac, Fairfield, NJ) filled with sugar or sugar plus 0.75 g microencapsulated retinyl palmitate (1,000,000 IU/g, Adisseo, Antony, France).

The steers were weighed monthly throughout backgrounding and finishing. At the start and end of finishing the steers were weighed again on consecutive days and ultrasound measurements were taken of backfat thickness and rib-eye area (Bergen *et al*. 1996).

4.3.2 Intensive Study Sub-population

A sub-population of five steers per genotype per treatment (n = 30) was randomly selected to perform more invasive procedures. At the start of finishing) and again at the end, a blood sample, liver biopsy, and subcutaneous fat biopsy were obtained from each animal in this sub-population. The start of finishing samples were obtained the day before the administration of the first vitamin A bolus and the end of finishing samples were obtained on the same day as (but prior to) administration of the final vitamin A bolus. This was performed to reduce the potential daily variation that a large monthly dose could have on the serum and liver vitamin A profiles.

The blood samples were collected via jugular veinipuncture into a 10 ml untreated (serum) Vacutainer tube (BD Diagnostics, Plymouth, UK) and immediately protected from light. The blood was analyzed for serum retinol concentration by Prairie Diagnostic Services (Saskatoon, SK).

The steers were injected intravenously with 0.1 mg/kg xylazine (Bayer, Toronto, ON) for sedation and intramuscularly with 1.5 ml/50 kg Anafen (ketoprofen; Merial, Baie D'Urfé, QC) for analgesia. Both biopsy sites were shaved and scrubbed three times with Baxedine (0.05% chlorhexidine gluconate; Omega, Montreal, QC) followed by 70% isopropanol (Rougier Pharma, Mirabel, QC), then injected subcutaneously with 6 ml 2% lidocaine (Bimeda MTC, Cambridge, ON). A skin biopsy near the tail head was made with an 8 mm biopsy punch (Acuderm Inc., Fort Lauderdale, FL) and the attached fat was collected and preserved in Allprotect Tissue Reagent (Qiagen, Mississauga, ON). A small incision was made between the 12^{th} and 13^{th} rib and a custom biopsy instrument with a 5 mm internal diameter was used to collect the liver specimen. A portion of the liver sample was preserved in RNAlater (Ambion, Austin, TX). The remainder

was protected from light, preserved on ice and shipped to the Diagnostic Center for Population and Animal Health (Michigan State University, Lansing, MI) where it was analyzed for vitamin A content.

4.4 Carcass Evaluation

The steers were processed in three groups over a two-week period at a commercial abattoir (Plains Processors Ltd., Carman, MB). Carcasses were evaluated for yield and quality grade within 72 hours post mortem by a trained grader according to Canadian Beef Grading Agency standards. A one-inch section of the *longissimus dorsi* (rib-eye steak) was collected from each carcass between the 12^{th} and 13^{th} rib and vacuum packaged for transport. Within 48 hours of collection the steaks were allowed to oxygenate (*i.e.* bloom) for 30 minutes at 4˚C and then assigned a marbling score according to the USDA ten-point scale by a trained grader.

The steaks were trimmed of intermuscular fat, vacuum packaged again, and stored at -20˚C until analyzed for intramuscular fat content. Each steak was thawed slightly and homogenized with a PowerPro food processor (Black and Decker, Towson, MD) and analyzed in duplicate for ether-extractable lipid content (intramuscular fat) by method 930.39(a) of the AOAC (1990). A 3 g sample was weighed into a cellulose extraction thimble (Whatman International Ltd., Maidstone, England), mixed with acid-washed
sand (Alfa Aesar, Heysham, England), and dried overnight at 105˚C. It was then extracted for 6 hours with petroleum ether (EMD, Gibbstown, NJ) on a Goldfisch apparatus (Labconco, Kansans City, MO). The ether was allowed to evaporate over night at room temperature and then the extracted fat was dried for 1 hour at 105˚C before weighing. Intramuscular fat content (IMF) was calculated as a percentage of the wet weight of the *longissimus dorsi*.

4.5 Gene Expression

The preserved fat and liver biopsies were flash frozen with liquid nitrogen and ground with a mortar and pestle. Centrifugation through a QIAshredder Mini Spin Column (Qiagen) further disrupted the tissue. RNA was extracted from liver using the RNeasy Mini Kit (Qiagen) and from fat with the RNeasy Lipid Tissue Mini Kit (Qiagen). A NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) was used to analyze RNA concentration and quality

(A260/A280 ratio). Total RNA was reverse transcribed to cDNA with oligo-dT and random primers using the QuantiTect Reverse Transcription Kit (Qiagen) and diluted to100 ng/µl.

Expression of *ADH1C* in liver and *fatty acid binding protein 4* (*FABP4*) in subcutaneous adipose tissue were assessed by qPCR. *ADH1C* was chosen as a target to assess if the *ADH1C c.-64T>C* SNP affects the expression of that gene. *FABP4* was chosen as a target because its expression has been associated with intramuscular fat content in cattle (Michal *et al*. 2006). Expression of both genes was quantified by qPCR using a standard curve over five points (100 to 0.001 pg/µl). Primers were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, IA) and obtained from Operon Biotechnologies Inc. The *ADH1C* PCR cocktail contained 10 µl KAPA SYBR FAST qPCR Master Mix (2X) ABI Prism (Kapa Biosystems, Woburn, MA) and 1000 nM of each primer (forward: CAGGACTACGAGAAACCCATC, reverse: TCCTACAATGACGCTTACACC). The *FABP4* cocktail contained 10 µl KAPA SYBR FAST qPCR Master Mix (2X) ABI Prism and 500 nM of each primer (forward: CTTAGATGAAGGTGCTCTGGTAC, reverse: GCTCTCTCATAAACTCTGGTGG). A StepOne Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) performed the following program for both genes: 10 minutes at 95˚C, 40 cycles of 15 seconds at 95˚C and 1 minute at 58˚C, and a melt curve from 60 to 95 ˚C at 0.3˚C intervals. Results were analyzed with StepOne Plus Real-Time PCR System software (Applied Biosystems) and reported as template copy number per ng cDNA (Staroscik 2004).

4.6 Statistical Analysis

All data were analyzed in a two (vitamin A supplementation) by three (*ADH1C c.-64T>C* genotype) factorial design as a two-way ANOVA using the mixed procedure of SAS 9.2 (SAS Institute, Inc.). Individual animals were used as the experimental unit. The effects of pen and kill date were included in the model as covariate but removed if not significant. Liver and serum retinol concentration were also analyzed by sampling time (start *vs.* end of finishing). Marbling score and IMF were analyzed for linear correlation. Significance was set at $P<0.05$ and trend at $P<0.10$. Means were separated using Tukey's LSD.

5 RESULTS

5.1 SNP Discovery

A total of thirteen SNPs and one insertion/deletion were discovered in the four candidate genes (Table 5.1). All of the SNPs were submitted to either Genbank or dbSNP, the SNP database of National Center for Biotechnology Information (NCBI 2010).

Table 5.1 SNPs discovered by sequencing *Bos taurus ADH1C*, *ALDH1A1*, *PPARγ*, and *RBP4* genes.

SNP	Location	Amino Acid Change	NCBI Submitted SNP Number[1]
ADH1C c.-64T>C	5' UTR	-	GQ862345[2]
ADH1C c.344T>A	Exon 4	Phe>Tyr	ss411633503
ADH1C c.415C>T	Exon 5	His>Tyr	ss411633504
ADH1C c.604T>G	Exon 6	-	ss411633505
ADH1C c.967C>T	Exon 8	-	ss411633508
ADH1C c.1072C>T	Exon 8	-	ss411633506
ALDH1A1 c.312G>A	Exon 3	-	ss411633507
ALDH1A1 c.1296A>T	Exon 11	-	ss411633511
*ALDH1A1 c.*27C>T*	3' UTR	-	ss411633509
*ALDH1A1 c.*109A>G*	3' UTR	-	ss411633510
PPARγ g.37441A>G	Intron 4	-	ss411633500
PPARγ c.1344G>T	Exon 7	Gln>His	ss411633512
PPARγ g.72132_g.72133insAT	Intron 6	-	ss411633501
RBP4 g.5470A>G	Intron 3	-	ss411633502

[1]Identification number assigned by dbSNP (NCBI 2010).
[2]Genbank accession number.

Six SNPs were found within *ADH1C*. The *c.-64T>C* SNP is located within a potential binding motif of the transcription factor C/EBPα. The C allele eliminates the CTTGA motif. Both the *c.344T>A* and *c.415C>T* SNPs result in the change of an amino acid, however in both cases the variant allele was present in only one of fourteen individuals. Both were in the same

individual (a Charolais cow) who was heterozygous at both loci, indicating that the alleles could be rare and in linkage disequilibrium. Both *c.604T>G* and *c.1072C>T* are silent mutations. The *c.967C>T* SNP is also silent but its location as the second base pair of exon 8 could potentially have an impact on splicing.

The *ALDH1A1* SNPs include two silent coding SNPs and two SNPs in the 3' UTR. The *c.312G>A* SNP is the last bp of exon 3, however the A allele was found in only one heterozygous individual (a Simmental x Belgian Blue cow) from a total of fourteen animals sequenced. The two 3' UTR SNPs, *c.*27C>T* and *c.*109A>G* could potentially impact mRNA stability.

One coding and two intronic SNPs were found in *PPARγ*. The *c.1344G>T* SNP is non-conserved as it changes glutamine, a polar amino acid, to histidine, a basic amino acid. Only one SNP was found in *RBP4*. The *g.5470A>G* SNP is located 27 bp from the end of intron 3.

5.2 SNP Selection

Five of the SNPs discovered from sequencing (*ADH1C* c.-64T>C, *ADH1C* c.967C>T, *ALDH1A1 c.*27C>T, ALDH1A1 c.*109A>G, PPARγ c.1344G>T*) were selected for further analysis based upon their potential for biological effects (*i.e.* changes in gene expression or protein structure) and favourable allele frequencies. *ADH1C c.-64T>C* removes a binding motif for a transcription factor, *ADH1C c.967C>T* is located near a splice junction and could interfere with splicing, *ALDH1A1 c.*27C>T* and *c.109A>G* are both located in the 3'UTR and could impact mRNA stability, and *PPARγ c.1344G>T* caused the change of an amino acid.

PCR-RFLP tests were designed to genotype cattle at each SNP. A population of 400 crossbred steers (described by Pugh 2007) were genotyped at each SNP and genotypes were analyzed for associations with production and carcass traits. The allele frequencies of the minor allele ranged between 0.05 for the T allele of *PPARγ c.1344G>T* to 0.30 for the C allele of *ADH1C c.-64 T>C* (Table 5.2).

The only significant association was between ALDH1A1 c.*27C>T and backgrounding average daily gain (Table 5.3). CC steers (n = 301) had significantly greater average daily gain (1.22 ± 0.010 lbs/day) than CT steers (1.15 ± 0.019 lbs/day; n = 88) but not TT steers (1.20 ± 0.079 lbs/day; n = 4).

Table 5.2 The allele frequencies of five SNPs in the Pugh 2007 steer population.

Allele Frequency	*ADH1C c.-64T>C*	*ADH1C c.967C>T*	*ALDH1A1 c.*27C>*	*ALDH1A1 c.*109A>G*	*PPARγ c.1344G>T*
Major Allele	T = 0.70	C = 0.92	C = 0.87	A = 0.90	G = 0.95
Minor Allele	C = 0.30	T = 0.08	T = 0.13	G = 0.10	T = 0.05

Table 5.3 The main effects of genotype at five SNPs with production and carcass traits in the Pugh 2007 steer population. Values in bold are significant (P<0.05).

Trait	*ADH1C c.-64T>C*	*ADH1C c.967C>T*	*ALDH1A1 c.*27C>T*	*ALDH1A1 c.*109A>G*	*PPARγ c.1344G>T*
Start of Test Weight	0.1412	0.2104	0.3264	0.7777	0.3034
End of Test Weight	0.4093	0.2454	0.4241	0.5196	0.7207
Shipping Weight	0.5062	0.2807	0.5186	0.5086	0.7133
Backgrounding ADG[1]	0.5478	0.1289	**0.0148**	0.6736	0.9799
Ultrasound Backfat	0.4997	0.6409	0.4987	0.9811	0.7635
Ultrasound Rib-eye Area	0.2741	0.3340	0.8130	0.3763	0.8655
Finishing ADG[1]	0.4731	0.5667	0.6461	0.8157	0.4132
Final Weight	0.5553	0.9957	0.4273	0.9244	0.4523
Warm Carcass Weight	0.5298	0.2466	0.9544	0.8739	0.4645
Average Fat	0.1922	0.3988	0.6218	0.7069	0.2953
Grade Fat	0.1235	0.5690	0.6757	0.7998	0.5058
Rib-eye Area	0.1926	0.1007	0.8921	0.2657	0.4783
Marbling Score	0.2355	0.7802	0.7081	0.8262	0.2099
Cutability	0.1743	0.7484	0.7307	0.6273	0.4459

[1]Average daily gain.

5.2.1 *Alcohol Deydrogenase 1C c.-64T>C*

ADH1C c.-64T>C was selected as the SNP to study in the subsequent nutrigenetic study. Although no significant associations were found within the Pugh (2007) 400 crossbred steer population, it was hypothesized that phenotypic differences would only be observed when vitamin A was limited (which it was not in this population). The removal of the motif for a transcription factor binding site gave it strong potential for reduced gene expression and it had the highest allele frequencies of the five SNPs examined (Table 5.2).

Populations of 50 animals from each of five major beef breeds were genotyped at the SNP by PCR-RFLP (Table 5.4). The allele frequencies of *ADH1C c.-64T>C* in the Charolais population were significantly different from the Simmental population, but not different between any of the other purebred populations studied (Table 5.4, $P<0.05$). Allele frequencies were not different between the Pugh (2007) and Vitamin A populations, with T allele frequency of 0.70 for both populations.

Table 5.4 Frequency of the *ADH1C c.-64T>C* T allele in five purebred (Angus, Charolais, Hereford, Limousin, and Simmental) cattle populations, where n is the population size.

Breed	n	T Allele Frequency
Angus	50	0.71^{ab}
Charolais	46	0.83^{a}
Hereford	50	0.76^{ab}
Limousin	50	0.73^{ab}
Simmental	48	0.66^{b}

[ab]Means not sharing a common superscript are significantly different ($P<0.05$).

5.3 Vitamin A Restriction Study

5.3.1 Serum and Liver Vitamin A

The interaction between vitamin A supplementation and sampling time (start *vs.* end of finishing) had a significant effect on both serum and liver vitamin A concentration (Table 5.5).

Table 5.5 The main effects of vitamin A supplementation, sampling time (start or end of finishing), and *ADH1C c.-64T>C* genotype on serum and liver vitamin A concentrations. Values in bold are significantly different (P<0.05).

Main Effect	Serum Vitamin A	Liver Vitamin A
Vitamin A	0.4259	0.6822
Sampling Time	**<0.0001**	0.1210
Genotype	0.2633	0.3715
Vitamin A x Sampling Time	**0.0451**	**0.0004**
Vitamin A x Genotype	0.8354	0.2025
Genotype x Sampling Time	0.9612	0.3124

At the start of finishing there was no difference in serum vitamin A levels between the two vitamin A treatments (Figure 5.1). Serum levels were significantly greater in both treatments by the end of finishing. Additionally, at the end of finishing the supplemented steers had significantly greater serum vitamin A than the unsupplemented steers.

Liver vitamin A concentration was significantly greater in unsupplemented steers than supplemented steers at the start of finishing (Figure 5.2). This pattern had reversed by the end of finishing, with the supplemented steers having greater liver vitamin A concentrations than the unsupplemented steers. Liver vitamin A was significantly higher at the start of finishing than at the end of finishing in unsupplemented steers but not different in supplemented steers.

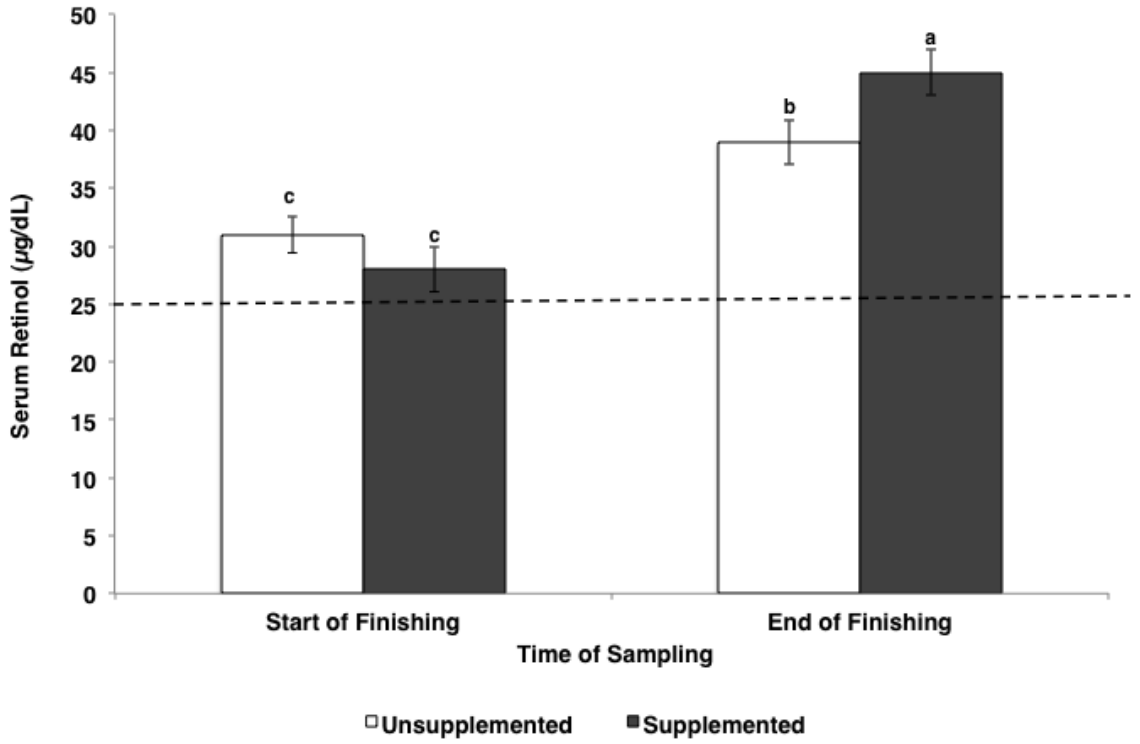

Figure 5.1 Serum vitamin A concentrations measured at the start and end of finishing from steers either unsupplemented or supplemented with vitamin A. Bars not sharing a common superscript are significantly different ($P<0.05$). The dashed line indicates the minimum normal serum vitamin A concentration for cattle (Radostits *et al*. 2000). Error bars depict the SEM.

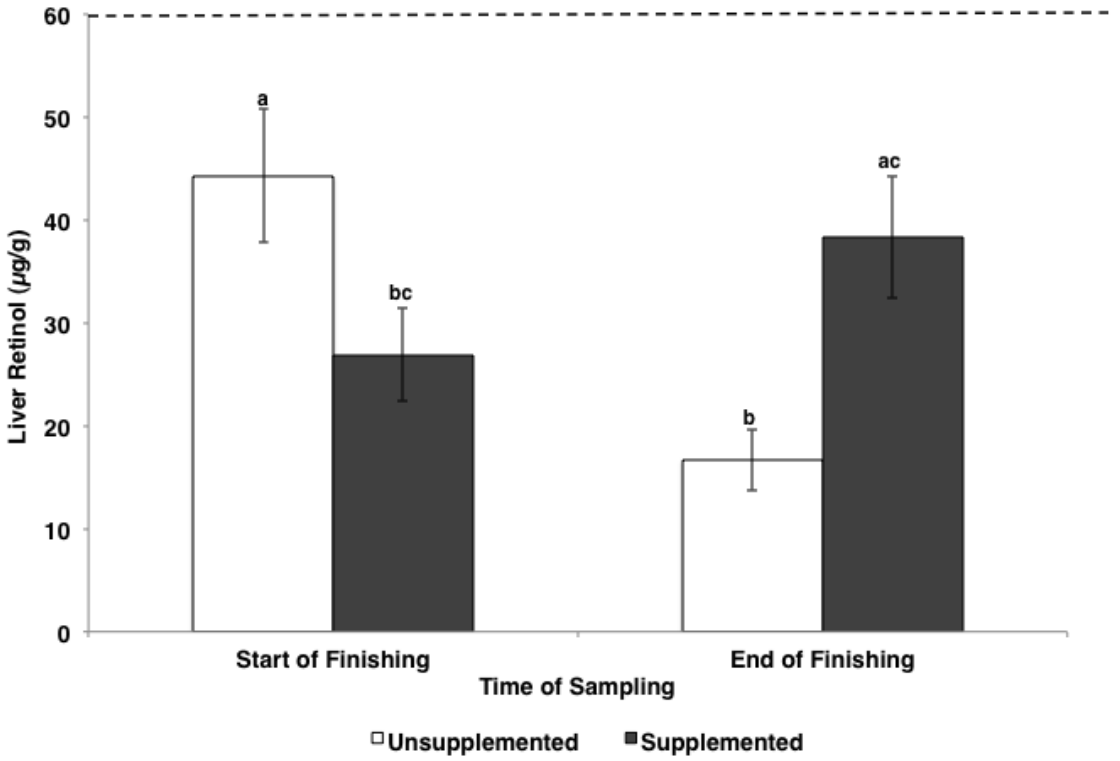

Figure 5.2 Liver vitamin A concentrations (wet weight) measured at the start and end of finishing from steers unsupplemented or supplemented with vitamin A. Bars not sharing a common superscript are significantly different ($P<0.05$). The dashed line indicates the minimum normal liver retinol concentration for cattle (Radostits *et al*. 2000). Error bars depict the SEM.

5.3.2 Production and Carcass Traits

Marbling score and IMF were highly correlated ($P<0.01$, $r = 0.7233$). Vitamin A treatment had a significant effect on both marbling score and IMF (Table 5.6). Marbling score was significantly greater for the unsupplemented than the supplemented steers, with scores of 5.7 ± 0.12 and 5.4 ± 0.12, respectively.

The interaction between vitamin A supplementation and *ADH1C c.-64T>C* genotype had a significant effect on IMF. Within the unsupplemented treatment, steers that were TT at the SNP had 22.9% greater IMF than CC steers (Figure 5.3). Additionally, unsupplemented TT steers had 24.4% greater IMF than supplemented TT steers.

Table 5.6 The effects of *ADH1C c.-64T>C* genotype, vitamin A supplementation, and their interaction on production and carcass traits. Values in bold are statistically significantly (P<0.05).

	Genotype			Vitamin A[1]			P Value		
Variable	TT	CT	CC	Unsupp	Supp	SEM	G	VA	G×VA
n	50	50	30	75	75				
Body Weight (kg)									
Start of BG[2]	307	306	305	305	307	1.8	0.90	0.61	0.59
Start of Finish	414	409	404	408	411	0.25	0.34	0.53	0.79
End of Finish	577	576	568	572	577	3.3	0.58	0.49	0.79
Ship	595	588	583	582	593	3.3	0.36	0.35	0.69
ADG (kg/day)									
BG[2]	0.99	0.97	0.94	0.98	0.96	0.016	0.49	0.49	0.73
Finish	1.32	1.32	1.28	1.30	1.32	0.013	0.34	0.63	0.73
Backfat[3] (mm)									
Start of Finish	4	4	4	4	4	0.2	0.73	0.14	0.92
End of Finish	11	11	11	11	11	0.2	0.81	0.77	0.15
Rib-eye Area[3] (cm^2)									
Start of Finish	53.68	54.97	53.24	53.99	54.16	0.411	0.21	0.84	0.98
End of Finish	82.07	82.39	80.17	81.36	82.12	0.583	0.33	0.52	0.48
Hot Carcass Wt (kg)	321	320	317	319	320	1.2	0.77	0.69	0.90
Dressing (%)	55.6	56.1	56.2	56.1	55.8	0.14	0.22	0.26	0.59
Average Fat (mm)	12	12	13	12	12	0.2	0.18	0.71	0.47
Grade Fat (mm)	10	11	12	11	11	0.2	0.12	0.76	0.98
Rib-eye Area (cm^2)	74	72	73	74	73	0.6	0.43	0.45	0.48
Lean Yield (%)[4]	56.1	55.7	55.1	55.9	55.5	0.24	0.33	0.45	0.52
Marbling Score[5]	5.6	5.4	5.7	5.7[a]	5.4[b]	0.08	0.54	**0.05**	0.16
Intramuscular Fat (%)	5.51	5.12	5.24	5.65[a]	4.96[b]	0.149	0.52	**0.02**	**0.011**

[1]Supp = supplemented with vitamin A, unsupp = no supplemental vitamin A
[2]BG = backgrounding
[3]Measured by ultrasonography.
[4]Calculated as 57.93 - 0.027(hot carcass wt) + 0.202(rib-eye area) - 0.703(average fat)
[5]Scores based up the USDA 10 point scale, where 1.0 is Devoid and 10.0 is Abundant.
[ab]Means within the same row not sharing a common superscript are significantly different (P<0.05).

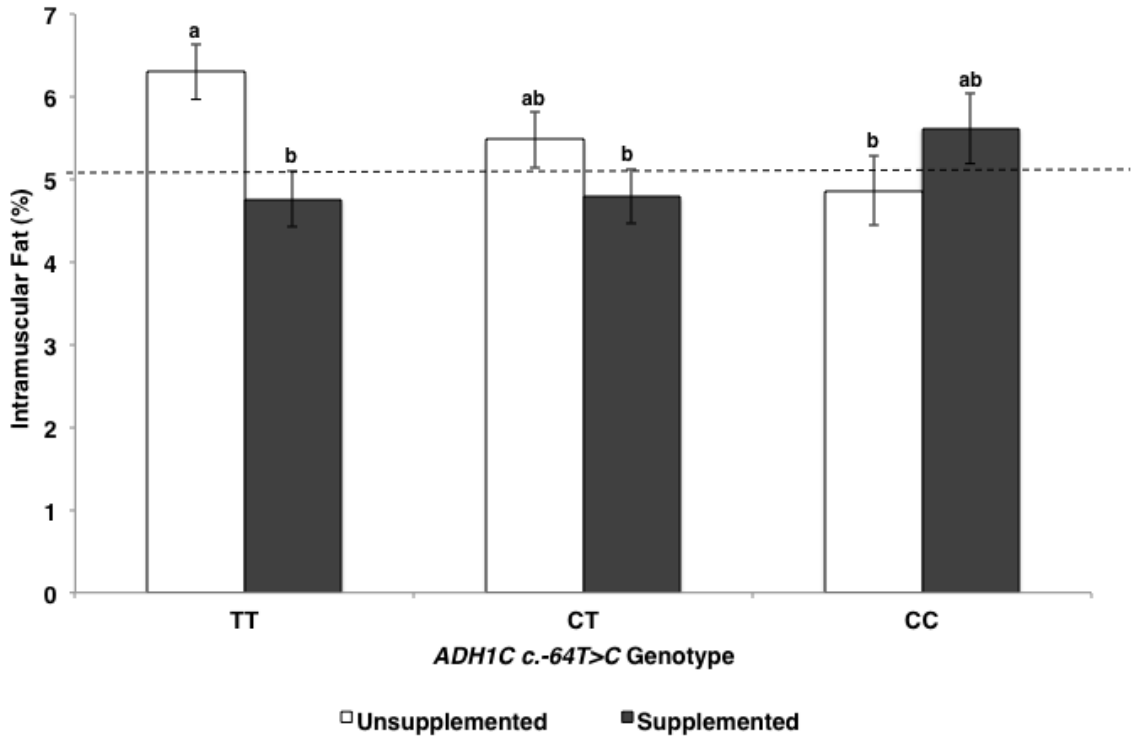

Figure 5.3 The interaction between vitamin A supplementation and *ADH1C c.-64T>C* genotype on the intramuscular fat content of the *longissimus dorsi* in finishing steers. Bars not sharing a common superscript are significantly different ($P<0.05$). The dashed line indicates the threshold between IMF associated with AA or AAA quality grades (Crews *et al*. 2010). Error bars depict the SEM.

5.3.3 Gene Expression

Vitamin A supplementation, *ADH1C c.-64T>C* genotype, and their interaction did not have a significant effect on *FABP4* expression in subcutaneous adipose tissue (Table 5.7). There was a significant effect of *ADH1C c.-64T>C* genotype on liver expression of *ADH1C*. TT steers expressed significantly more *ADH1C* mRNA than CC steers, and CT steers were intermediate (Table 5.8).

Table 5.7 The main effects of *ADH1C c.-64T>C* genotype, vitamin A supplementation, and their interaction on expression of *ADH1C* mRNA in liver and *FABP4* mRNA in fat. Values in bold are significant (P<0.05).

Main Effect	*ADH1C*	*FABP4*
Vitamin A	0.3387	0.6462
Genotype	**0.0009**	0.9774
Interaction	0.3746	0.7878

Table 5.8 Expression of *ADH1C* mRNA in liver from steers of each *ADH1C c.-64T>C* genotype.

Genotype	Expression (template copies/ng cDNA)
TT	219[a]
CT	155[ab]
CC	79[b]
SEM	59.3

[ab]Means not sharing a common superscript are significantly different (P<0.05).

6 DISCUSSION

6.1 SNP Selection

The five SNPs genotyped in the Pugh (2007) crossbred steer population were selected based upon their potential to alter either gene expression or the resultant protein. The C allele of *ADH1C c.-64T>C* removes a potential binding site for the transcription factor C/EBPα, which could reduce the expression of *ADH1C* and result in lower levels of the ADH1C enzyme compared to the T allele.

ADH1C c.967C>T is the second bp of exon 8. Its location adjacent to a splice junction could potentially interfere with splicing, resulting in exon skipping or partial intron inclusion in the mature mRNA. This would cause the production of a truncated protein (if a premature stop codon is introduced) or significant changes in the protein sequence, both of which can result in a non-functional protein.

*ALDH1A1 c.*27C>T* and *ALDH1A1 c.*109A>G* are both located in the 3' UTR of the expressed mRNA. Mutations in this region have been shown to alter mRNA stability and rate of translation (reviewed by Grzybowska *et al*. 2001). Both of these mechanisms can lead to a reduction in the amount of protein produced.

Lastly, *PPARγ c.1344G>T* results in the non-conserved amino acid substitution of glutamine to histidine. This could alter the folding of the protein and subsequently reduce or eliminate the ability of PPARγ to activate adipogenic genes.

ADH1C c.344T>A and *c.415C>T* both result in the change of an amino acid, conserved and non-conserved respectively. *ALDH1A1 c.312G>A,* though a silent mutation, could interfere with splicing because of its location at a splice junction as the last bp of exon 3. These three SNPs were not selected for further analysis due to the likelihood that the rare allele would have very low allele frequencies (in each instance only one heterozygous animal was present among the population used for sequencing).

The SNP selected for use in the vitamin A restriction study was *ADH1C c.-64T>C*. The C/EBP family of transcription factors has been shown in humans to control expression of *ADH1* in the liver (Stewart *et al*. 1991). The removal of the binding site for C/EBPα by the C allele of *ADH1C c.-64T>C* could therefore reduce the liver expression of *ADH1C* in cattle (Figure 6.1). This SNP also had the most favourable allele frequency of the five SNPs examined (Table 5.2);

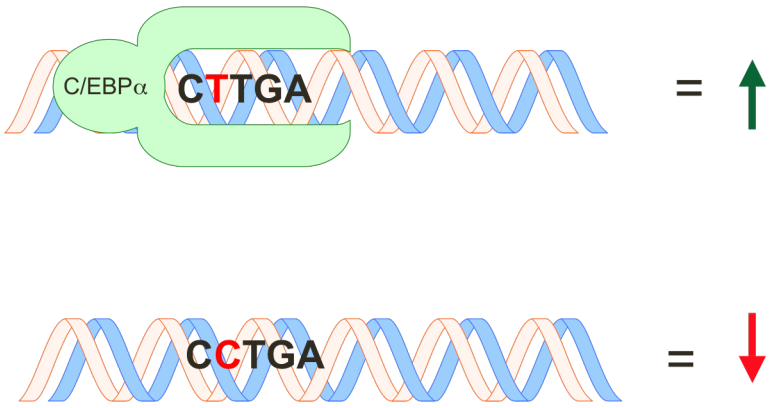

Figure 6.1 Illustration of the proposed enhancement of *ADH1C* expression by the transcription factor C/EBPα. The T allele of *ADH1C c.-64T>C* creates a potential binding motif for C/EBPα, where as the C allele disrupts the motif.

it was the only SNP that was feasible to obtain sufficient animals of the rare genotype to proceed with the study. As such, only 31 steers of the CC genotype were present in the initial population of 450 animals obtained for the vitamin A restriction study.

The allele frequencies for the SNP were similar for all breeds studied, with the exception of the Charolais population that was significantly different from the Simmental population but not the other three beef breeds tested (Table 5.5). It is not clear why the frequency of the T allele would be higher in the Charolais than other beef breeds. It could be an artifact of the small number of animals tested, or it is possible that the T allele is in linkage disequilibrium with an allele that was more strongly selected for in Charolais than in other beef breeds.

The *ADH1C c.-64T>C* SNP did not have a significant effect on carcass or production traits in the Pugh 2007 steer population (Table 5.4). It was hypothesized that a difference in phenotype would only be observed when vitamin A was limited. These steers were supplemented with vitamin A in their diet (Pugh 2007), which explains why there was no significant effect of the SNP observed. Additionally, data were only available for marbling score, a subjective measurement, and not IMF, an objective measurement of the fat content of the meat.

6.2 Vitamin A Restriction Study

6.2.1 Serum and Liver Vitamin A

At the start of finishing there was no difference in serum vitamin A concentration between steers in either vitamin A treatment group (Figure 5.1). This was anticipated as all steers had received the same vitamin A-deficient backgrounding diet (Table 4.3) up to this point. It is unclear why at the start of finishing the unsupplemented steers had significantly greater liver vitamin A than supplemented steers. Animals were randomly allocated to vitamin A treatment group and all biopsied steers, regardless of treatment, were housed in the same pen and fed the same diet. Although the values are different, they are both substantially lower than the minimum normal threshold of 60 µg/g for liver vitamin A (Radostits *et al*. 2000), indicating that the steers' vitamin A stores had been depleted. This primed the animals to respond to the vitamin A treatments during finishing.

Serum vitamin A was significantly greater at the end of finishing than at the start of finishing for steers of both vitamin A treatments. In contrast, liver vitamin A was not different between start and end of finishing in the supplemented steers but was significantly lower at the end of finishing for the unsupplemented steers (Figure 5.2). The liver levels remained below normal (60 µg/g; Radostits *et al*. 2000) for both vitamin A treatments. Though liver vitamin A stores decreased in the unsupplemented steers, the serum levels remained within the normal range of 25 to 60 µg/dL (Radostits *et al*. 2000), indicating that the animals had not developed vitamin A deficiency.

Serum vitamin A was significantly higher in the supplemented steers than the unsupplemented steers at the end of finishing. This was also reflected in the liver, where the supplemented steers had significantly greater liver vitamin A than the unsupplemented steers at the end of finishing. Bryant *et al*. (2010) found a significant difference in liver retinol concentration in as little as 56 days between steers that received either 0 or 2205 IU/kg DM supplemental vitamin A. Additionally, Arnett *et al*. (2009) found a significant difference in serum vitamin A concentration between supplemented and unsupplemented steers within 45 days on feed. Our results indicate that the treatments were successful in producing a difference in vitamin A status between the two groups by the end of the finishing period.

6.2.2 Production and Carcass Traits

No effect of *ADH1C* genotype, vitamin A supplementation, or their interaction was found on the production traits measured (Table 5.6). Several previous studies have also found no association between vitamin A supplementation and production traits such as feed intake, average daily gain, and final weight (Pyatt *et al*. 2005, Gorocica-Buenfil *et al*. 2008, Kruk *et a*l. 2008, Arnett *et al.* 2009, Pickworth *et al.* 2009). In studies where effects on production traits were found, the differences were attributed to the steers that received the lowest vitamin A treatments entering subclinical vitamin A deficiency (Oka *et al.* 1998, Oka *et al*. 2004, Wang *et al.* 2007). One of the first symptoms of vitamin A deficiency in cattle is a reduction in dry matter intake (Frey *et al.* 1947, NRC 1987), which can explain the lower ADG and carcass weight associated with vitamin A restriction observed in these studies. In all three studies, the cattle received straw-based diets (nearly devoid of vitamin A precursors) for six to thirteen months, leading to subclinical vitamin A deficiency.

The unsupplemented steers had significantly greater marbling scores than those that were supplemented (Table 5.6). Several other studies have also reported significant increases in marbling score in cattle when vitamin A was restricted. Wang *et al*. (2007) found an inverse relationship between marbling and level of vitamin A supplementation. Pickworth *et al*. (2009) reported that steers fed no supplemental vitamin A had a greater proportion grading USDA Choice (Canada AAA) or greater than steers receiving 3,750 IU/kg DM supplemental vitamin A.

The interaction between *ADH1C* genotype and vitamin A level had a significant effect on IMF. When vitamin A was not supplemented, TT steers had on average 6.30 % IMF (Figure 5.3), which is associated with Canada AAA and USDA Choice quality grade (Crews *et al*. 2010). In contrast, unsupplemented CC steers had on average 4.86% IMF, which is associated with Canada AA and USDA Select quality grades. The mean IMF of all three genotypes of supplemented steers were within the range associated with Canada AA and USDA Select (Crews *et al*. 2010). The increase in IMF observed in unsupplemented TT steers could have a significant impact on feedlot profitability, as higher grading carcasses (*i.e.* AAA and Prime) receive a premium when marketed on a marbling grid (DiCostanzo and Dahlen 2000).

No significant interaction was found between *ADH1C* genotype and vitamin A level on marbling score (Table 5.6). Marbling score and IMF were strongly correlated ($P<0.01$, $r =$

0.7233), which is in agreement with Savell *et al*. (1986), who reported a significant regression between USDA marbling score and IMF. Therefore it is likely that with a larger population of cattle, such as a commercial feedlot, the interaction between *ADH1C* genotype and vitamin A level would be significant for both marbling score and IMF.

6.2.3 Gene Expression

FABP4 is expressed in mature adipocytes (Urs *et al*. 2004, Pickworth *et al*. 2011) and it has been associated with IMF in cattle (Michal *et al*. 2006). The vitamin A pathway promotes adipogenesis through adipocyte maturation (Ziouzenkova *et al.* 2007, Smith *et al.* 2009), therefore it was hypothesized that because the unsupplemented TT steers had greater IMF that they would also have a greater proportion of mature adipocytes in their fat biopsies, which would be reflected by greater expression of *FABP4*. It is possible that no differences were seen in *FABP4* expression (Table 5.7) because subcutaneous fat is an early depot for adipose accumulation (Cianzio *et al.* 1985) and the time of sampling was too late to detect differences in adipocyte differentiation. By the time of biopsy all steers would have subcutaneous adipose tissue consisting of primarily mature adipocytes. It is possible that differences in *FABP4* expression could be detected in intramuscular adipose tissue, as it is the last fat depot to be formed in finishing (Cianzio *et al.* 1985). There are conflicting reports in regards to associations between *FAPB4* expression in intramuscular adipose tissue and marbling. Wang *et al.* (2009) found greater expression in Wagyu x Hereford (high marbling) than Piedmontese x Hereford (lower marbling) cattle, however Pickford *et al.* (2011) found no difference between high IMF and low IMF steers.

Hepatic expression of *ADH1C* mRNA was significantly greater in TT steers than CC steers, with CT steers being intermediate (Table 5.8). The SNP is located within a potential binding site motif for the transcription factor C/EPBα (Chekmenev *et al.* 2005). The C allele eliminates the motif, potentially reducing transcription of the gene, resulting in the observed reduction in mRNA levels. In humans C/EBP has been implicated in the regulation of *ADH1* in the liver (Stewart *et al*. 1991). Further study, such as chromatin immunoprecipitation, is required to confirm that the *c.-64T>C* SNP does in fact alter C/EBPα binding within the promoter of *ADH1C*.

It is possible that reduced expression of *ADH1C* further translates into lower levels of the ADH1C enzyme in cattle with the C allele. When vitamin A is supplied in excess of minimum requirements, such as the supplemented treatment, it is likely that steers of each genotype can adequately metabolize retinol to retinaldehyde and subsequently retinoic acid and maintain optimum levels of both metabolites.

When retinol is limited, as was the case with the unsupplemented treatment, it can be assumed that there is a reduction in retinaldehyde production in all steers, regardless of genotype. If CC steers produce less ADH1C, it is possible that they experience an even greater reduction in retinaldehyde production than TT steers (Figure 6.2). High levels of unbound cellular retinol binding protein, which binds retinaldehyde within the cell, promotes mobilization of retinyl stores, maintaining retinoic acid biosynthesis (Napoli 1996). Additionally, the activity of ALDH1A1, which catalyzes the conversion of retinaldehyde to retinoic, increases three-fold during vitamin A deficiency (Napoli *et al.* 1995). Therefore it is likely that when retinol is limiting, retinoic acid levels are maintained at the expense of the intermediary retinaldehyde. We postulate that when vitamin A is restricted, both TT and CC steers experience a reduction in retinaldehyde levels. TT steers are able to maintain similar levels of retinoic acid as when vitamin A is plentiful, but CC steers, due to their greater reduction in retinaldehyde synthesis, also incur a reduction in retinoic acid synthesis.

Retinoic acid has been shown to be a potent stimulator of adipogenesis, whereas retinaldehyde is antagonistic and inhibits adipogenesis (Ziouzenkova *et al.* 2007). The balance between retinaldehyde and retinoic acid is a major factor controlling intramuscular adipogenesis. In the unsupplemented TT steers, the retinoic acid levels remained adequate to stimulate adipogenesis and the lower levels of retinaldehyde reduced the inhibition of adipogenesis; the net result being an increase in IMF. In the unsupplemented CC steers, although the inhibition by retinaldehyde was also reduced, there was insufficient retinoic acid to maximize stimulation of adipogenesis. The net result was no change in IMF compared to steers supplemented with vitamin A.

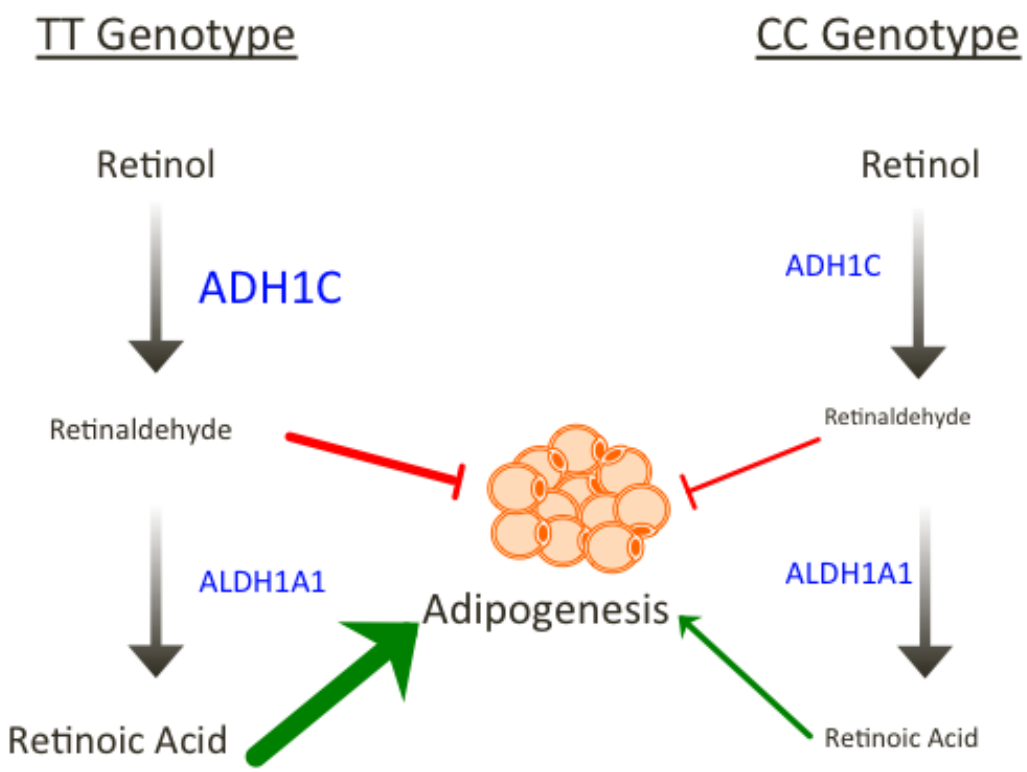

Figure 6.2 Diagrammatic representation of the proposed mechanism for the difference in adipogenesis observed in cattle of different *ADH1C c.-64T>C* genotypes when vitamin A is restricted. Cattle of the CC genotype produce less alcohol dehydrogenase 1C (ADH1C) than TT cattle, and subsequently less retinaldehyde when retinol is limited. Retinaldehyde inhibits adipogenesis (red lines) and is metabolized by aldehyde dehydrogenase 1A1 (ALDH1A1) to retinoic acid, which stimulates adipogenesis (green lines).

6.3 Future Research

This project was an initial proof of concept study, and therefore leaves room for further investigation. In an effort to maximize the potential for marbling, black Angus cross steers were used and they were not given hormonal implants. Before the results of this research can be applied to the beef industry it is important to validate the findings of this research in commercial beef cattle (comprised of a variety of breed crosses) and with the use of hormonal implants.

The backgrounding diet used in the vitamin A restriction study was specifically formulated to be low in β-carotene. To accomplish this, atypical ingredients such as oat hulls and barley straw comprised a significant portion of the diet. If future studies are performed, they should use a

backgrounding diet that reflects industry practices. Additionally, this study only examined two levels of vitamin A supplementation: 0 IU/month (unsupplemented) and 750,000 IU/month (supplemented), which was an approximation of NRC (1996) recommendations. Further research is required to determine the optimum level of vitamin A supplementation to maximize IMF.

Although the interaction between *ADH1C c.-64T>C* genotype and vitamin A supplementation was significantly associated with differences in IMF, and a biochemical mechanism has been proposed to explain the observed effect, further research is required to prove that *ADH1C c.-64T>C* is the causative mutation. Chromatin immunoprecipitation could be used to determine if C/EBPα binds to the motif surrounding the T allele but not the C allele of the SNP. The amount of ADH1C enzyme and/or its activity could be measured in cattle of each genotype. Lastly, the concentrations of retinaldehyde and retinoic acid could be measured in intramuscular adipose tissue in cattle of each genotype after vitamin A restriction to determine if the proposed mechanism is valid.

7 CONCLUSIONS

The purpose of this project was to discover if a polymorphism within a gene involved in the vitamin A metabolism pathway is associated with the previously observed increase in marbling when vitamin A supplementation is reduced. We have demonstrated that the TT genotype at *ADH1C c.-64T>C* in combination with vitamin A restriction is associated with significantly increased IMF in beef cattle.

The use of *ADH1C c.-64T>C* in a marker-assisted management program has the potential to significantly improve the profitability of a feedlot that markets their cattle on the grid system. With a T allele frequency of 0.70 in crossbred cattle, nearly half of the cattle in a commercial feedlot would be the TT genotype. If cattle were sorted by genotype and fed a low vitamin A diet, the TT cattle would deposit more IMF in a given amount of time and therefore could be marketed earlier than CT and CC cattle, substantially reducing feed costs. Alternatively, if all cattle were marketed at the same time TT cattle would have more IMF, and as IMF is strongly correlated to marbling score, more TT cattle would reach higher quality grades, for which premiums are paid.

There is also potential for cow-calf producers to profit from the SNP. The Genetic Breeder Alliance of Cattleland Feedyards (Strathmore, AB) offers producers enrolled in the program a premium of $0.03/lb for calves that are genotyped TT or CT at *leptin R25C* (Furber 2011). If a similar program was implemented for the *ADH1C* SNP, cow-calf producers would have an incentive to select for the T allele and share in the increased profit that feedlots receive from the TT genotype.

8 REFERENCES

Alexander, G., Bennett, J.W., and Gemmell, R.T. 1975. Brown adipose tissue in the new-born calf (*Bos taurus*). J. Physiol. **244**: 223-234.

Altshuler, D., Pollara, V.J., Cowles, C.R., Van Etten, W.J., Baldwin, J., Linton, L., and Lander, E.S. 2000. An SNP map of the human genome generated by reduced representation shotgun sequencing. Nature **407**: 513-516.

Anderson K.B., Nilsson, A., Blomhoff, H.K., Øyen, T.B., Gabrielsen, O.S., Norum, K.R., and Blomhoff, R. 1992. Direct mobilization of retinol from hepatic perisinusoidal stellate cells to plasma. J. Biol. Chem. **267**: 1340-1344.

AOAC. 1990. Official methods of analysis. 15th ed. Assoc. Offic. Anal. Chem., Washington, DC.

Appling, D.R. and Chytil, F. 1981. Evidence of a role for retinoic acid (vitamin A-acid) in the maintenance of testosterone production in male rats. Endocrinology **108**: 2120-2123.

Arnett, A.M., Dikeman, M.E., Daniel, M.J., Jaeger, J., and Perrett, J. 2009. Effects of vitamin A supplementation and weaning age on serum and liver retinol concentrations, carcass traits, and lipid composition in market beef cattle. Meat Sci. **81**: 596-606.

Bardot, O., Aldridge, T.C., Latruffe, N., and Green, S. 1993. PPAR-RXR heterodimer activates a peroxisome proliferators response element upstream of the bifunctional enzyme gene. Biochem. Biophys. Res. Commum. **192**: 37-45.

Bates, C.J. 1983. Vitamin A in pregnancy and lactation. Proc. Nutr. Soc. **42**: 65-79.

Bergen, R.D., McKinnon, J.J., Christensen, D.A., and Kohle, N. 1996. Prediction of lean yield in yearling bulls using real-time ultrasound. Can. J. Anim. Sci. **75**: 305-311.

Berni, R., Clerici, M., Malpeli, G., Cleris, L., and Formelli, F. 1993. Retinoids: in vitro interaction with retinol-binding protein and influence on plasma retinol. FASEB J. **7**: 1179-1184.

Blackfan, K.D. and Wolbach, S.B. 1933. Vitamin A deficiency in infants: a clinical and pathological study. J. Pediatr. **3**: 679-706.

Blanco, E., Messeguer, X., Smith, T.F., and Guigó, R. 2006. Transcription factor map alignment of promoter regions. PLoS Comput. Biol. **2**: e49.

Blomhoff, R., Eskild, W., Kindberg, G.M., Prydz, K., and Berg, T. 1985. Intracellular transport of endocytosed chylomicron [^{3}H]retinyl ester in rat liver parenchymal cells.

Evidence for translocation of a [^{3}H]retinoid from endosomes to endoplasmic reticulum. J. Biol. Chem. **260**: 13566-13570.

Blomhoff, R., Green, M.H., Berg, R., and Norum, K.R. 1990. Transport and storage of vitamin A. Science **250**: 399-404.

Blomhoff, R., Helgerud, P., Rasmussen, M., Berg, T., and Norum, K.R. 1982. In vivo uptake of chylomicron [^{3}H]retinyl ester by rat liver: evidence for retinol transfer from parenchymal to non parenchymal cells. PNAS **79**: 7326-7330.

Boleda, M.D., Saubi, N., Farrés, J., and Parés, X. 1993. Physiological substrates for rat alcohol dehydrogenase classes: aldehydes of lipid peroxidation, ω-hydroxyfatty acids, and retinoids. Ach. Biochem. Biophys. **307**: 85-90.

Bonet, M.L., Ribot, J., Felipe, F., and Palou, A. 2003. Vitamin A and the regulation of fat reserves. Cell. Mol. Life Sci. **60**: 1311-1321.

Britton. G. 1995. Structure and properties of carotenoids in relation to function. FASEB J. **9**: 1551-1558.

Bryant, T.C., Wagner, J.J., Tatum, J.D., Galyean, M.L., Anthony, R.V., and Engle, T.E. 2010. Effect of dietary supplemental vitamin A concentration on performance, carcass merit, serum metabolites, and lipogenic enzyme activity in yearling beef steers. J. Anim. Sci. **88**: 1463-1478.

Buchanan, F.C., Van Kessel, A.G., Boisclair, Y.R., Block, H.C., and McKinnon, J.J. 2007. The leptin arg25cys affects performance, carcass traits and serum leptin concentrations in beef cattle. Can. J. Anim. Sci. **87**: 153-156.

Bugge, T.H., Pohl, J., Lonnoy, O., and Stunnenberg, H.G. 1992. RXR alpha, a promiscuous partner of retinoic acid and thyroid hormone receptors. EMBO J. **11**: 1409-1418.

Canada Beef Export Federation. 2009. Comparisons to the U.S.D.A. (American) grading system. Accessed August 19, 2011. http://www.cbef.com/beefquality.htm#qualitygrade

Canadian Council on Animal Care. 1993. Guide to the care and use of experimental animals. Vol. 1. Can. Counc. Anim. Care, Ottawa, ON.

Cartegni, L., Chew, S.L., and Krainer, A.R. 2002. Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat. Rev. Genet. **3**: 285-298.

Chambon, P. 1996. A decade of molecular biology of retinoic acid. FASEB J. **10**: 940-954.

Chekmenev, D.S., Haid, C., and Kel, A.E. 2005. P-Match: transcription factor binding site search by combining patterns and weight matrices. Nucleic Acids Res. **33**: W432-W437.

Chen, J.M., Férec. C., and Cooper, D.N. 2006. A systematic analysis of disease-associated variants in the 3' regulatory regions of human protein-coding genes II: the importance of mRNA secondary structure in assessing the functionality of 3' UTR variants. Hum. Genet. **120**: 301-333.

Chew, B.P. 1993. Effects of supplemental b-carotene and vitamin A on reproduction in swine. J. Anim. Sci. **71**: 247-252.

Cianzio, D.S., Topel, D.G., Whitehurst, G.B., Beitz, D.C., and Self, H.L. 1982. Adipose tissue growth in cattle representing two frame sizes: distributions among depots. J. Anim. Sci. **55**: 305-312.

Cianzio, D.S., Topel, D.G., Whitehurst, G.B., Beitz, D.C., and Self, H.L. 1985. Adipose tissue growth and cellularity: changes in bovine adipocyte size and number. J. Anim. Sci. **60**: 970-976.

Cornelius, P., MacDougald, O., and Lane, M.D. 1994. Regulation of adipocyte development. Annu. Rev. Nutr. **14**: 99-129.

Cousin, B.L., Casteilla, L., Lafontan, M., Ambid, L., Langin, D., Berhault, M.F., and Penicaud, L. 1993. Local sympathetic denervation of white adipose tissue in rats induces preadipocyte proliferation without noticeable changes in metabolism. Endocrinology **133**: 2255-2262.

Crews, D., Dikeman, M., Northcut, S.L., Garrick, D., Marston, T.T., MacNeil, M., Olson, L.W., Paschal, J.C., Rouse, G., Weaber, B., Wheeler, T., Shackelford, S., Williams, R.E., and Wilson, D. 2010. Animal evaluation, Page 36 *in* L.V. Cundiff, L.D. Van Vleck, and W.D. Hohenboken, eds. Guildlines for Uniform Beef Improvement Programs. 9th ed. Beef Improvement Federation, Raleigh, NC.

Cullison, A.E. and Ward, C.S. 1965. Costal Bermudagrass hay as a source of vitamin A for beef cattle. J. Anim. Sci. **24**: 969-972.

Cusack. P.M.V., McMeniman, N.P., and Lean, I.J. 2008: Effects of injectable vitamins A, D, E and C on the health and growth rate of feedlot cattle destined for the Australian domestic market. Aust. Vet. J. **86:** 81-87.

Dawkins, M.J.R. and Hull, D. 1963. Brown fat and the response of the new-born rabbit to cold. J. Physiol **169 (Suppl.)**: 101P.

Desvergne, B. 2007. RXR: from partnership to leadership in metabolic regulations. Vitam. Horm. **75**: 1-35.

DiCostanzo, A. and Dahlen, C.R. 2000. Grid pricing as a fed cattle marketing strategy. Minnesota Cattle Feeder Report B-470. University of Minnesota, St. Paul, MN.

DiRenzo, J., Söderström, M., Kurokawa, R., Ogliastro, M. H., Ricote, M., Ingrey, S., Hörlein, A., Rosenfeld, M.G., and Glass, C.K. 1997. Peroxisome proliferators-activated receptors and retinoic acid receptors differentially control the interactions of retinoid X receptor heterodimers with ligands, coactivatrors, and corepressors. Mol. Cell. Biol. **17**: 2166-2176.

Duan, J., Wainright, M.S., Comeron, J.M., Saitou, N., Sanders, A.R., Gelernter, J., and Gejman, P.V. 2003. Synonymous mutations in the human *dopamine receptor D2* (*DRD2*) affect mRNA stability and synthesis of the receptor. Human Mol. Genet. **12**: 205-216.

Duckett, S.K., Wagner, D.G., Owens, F.N., Dolezal, H.G., and Gill, D.R. 1999. Effect of anabolic implants on beef intramuscular lipid content. J. Anim. Sci. **77**: 1100-1104.

Duester, G. 2000. Families of retinoid dehydrogenases regulating vitamin A function: production of visual pigment and retinoic acid. Eur. J. Biochem. **267:** 4315-4324.

Duester, G., Farrés, J., Felder, M.R., Holmes, S.R., Höög, J.O., Parés, X., Plapp. B.V., Yin, S.J., and Jörnvall, H. 1999. Recommended nomenclature for the vertebrate alcohol dehydrogenase gene family. Biochem. Pharmacol. **58**: 389-395.

FAO. 1967. Availability of carotene from various sources. Pages 74-79 *in* Requirements of vitamin A, thiamine, riboflavin and niacin: report of a joint FAO/WHO expert group. FAO, Rome, Italy.

Fes, G., and Johannesson, G. 1987. Studies of the spontaneous transfer of retinol from the retinol:retinol-binding protein complex to unilamellar liposomes. Biochim. Biophys. Acta **901**: 255-264.

Frey, P.R., Jensen, R., and Connell, W.E. 1947. Vitamin A intake in cattle in relation to hepatic stores and blood levels. J. Nutr. **34**: 421-430.

Fu, M., Sun, T., Bookout, A. L., Downes, M., Yu, R. T., Evand, R. M., and Mangelsdorf, D. J. 2005. A nuclear receptor atlas: 3t3-L1 adipogenesis. Mol. Endocrinol. **19**: 2437-2450.

Furber, D. 2011. Feeding by the genes. Cattleman. **March 14th**: 22-23.

Gaskins, H.R., Kim, J.W., Wright, J.T., Rund, L.A., and Hausman, G.J. 1990. Regulation of insulin-like growth factor-I ribonucleic acid expression, polypeptide secretion, and binding protein activity by growth hormone in porcine preadipocyte cultures. Endocrinology **126**: 622-630.

Goodman, D.S. 1984. Plasma retinol-binding protein. Pages 42-88 *in* Sponr, M.B., Roberts, A.B., and Goodman, D.S., eds. The Retinoids. Vol. 2. Academic Press, Orlando, Florida.

Goodman, D.S. and Olson, J.A. 1969. The conversion of all trans β-carotene into retinal. Methods Enzymol. **15**: 462-475.

Gorocica-Buenfil, M.A., Fluharty, F.L., Bohn, T., Schwartz, S.J., and Loerch, S.C. 2007a. Effect of low vitamin A diets with high-moisture or dry corn on marbling and adipose tissue fatty acid composition of beef steers. J. Anim. Sci. **85:** 3355-3366.

Gorocica-Buenfil, M.A., Fluharty, F.L., Reynolds, C.K., and Loerch, S.C. 2007b. Effect of dietary vitamin A restriction on marbling and conjugated linoleic acid content in Holstein steers. J. Anim. Sci. **85:** 2243-2255.

Gorocica-Buenfil, M.A., Fluharty, F.L., and Loerch, S.C. 2008. Effect of vitamin A restriction on carcass characteristics and immune status of beef steers. J, Anim. Sci. **86:** 1609-1616.

Grzybowska, E.A., Wilczynska, A., and Siedlecki, J.A. 2001. Regulatory functions of the 3'UTRs. Biochem. Biophys. Res. Commun. **288**: 291-295.

Guilbert, G.R. 1935. Factors affecting the carotene content of alfalfa hay and meal. J. Nutr. **10**: 45-62.

Harrison, E.H. 2005. Mechanisms of digestion and absorption of dietary vitamin A. Annu. Rev. Nutr. **25**: 87-103.

Harrison, E.H. and Gad, M.Z. 1989. Hydrolysis of retinyl palmitate by enzymes of rat pancreas and liver. Differentiation of bile salt-dependent and bile salt-independent, neutral retinyl ester hydrolases in rat liver. J. Biol. Chem. **264**: 17142-17147.

Hausman, D.B., DiGirolamo, M., Bartness, T.J., Hausman, G.J., and Martin, R.J. 2001. The biology of white adipose proliferation. Obes. Rev. **2**: 239-254.

Hausman, D.B., Dodson, M.V., Ajuwan, K., Azain, M., Barnes, K.M., Guan, L.L., Jiang, Z., Poulos, S.P., Sainz, R.D., Smith, S., Spurlock, M., Novakofski, J., Fernyhoughm M.E., and Bergen, W.G. 2009. The biology and regulation of preadipocytes and adipocytes in meat animals. J. Anim. Sci. **87**: 1218-1246.

Hausman, G.J., Wright, J.T., Dean, R., and Richardson, R.L. 1993. Cellular and molecular aspects of the regulation of adipogenesis. J. Anim. Sci. **71 (Suppl. 2)**: 33-55.

Heaton, J.M. 1972. The distribution of brown adipose tissue in the human. J. Anat. **112**: 35-39.

Hentze, M.W. and Kulozik, A.E. 1999. A perfect message: RNA surveillance and nonsense-mediated decay. Cell **96**: 307-310.

Heyman, R.A., Mangelsdorf, D.J., Dyck, J.A., Stein, R.B., Eichele, G., Evans, R.M., and Thaller, C. 1992. 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell **68**: 397-406.

Higdon, J. 2003. Vitamin A. Micronutrient Information Center. Linus Pauling Institute, Oregon State University. [http://lpi.oregonstate.edu/infocenter/vitamins/vitaminA/rxr.html/]. Accessed October 7th, 2008.

Högström, M., Nordström, A., and Nordström, P. 2008. Retinol, retinol-binding protein 4, abdominal fat mass, peak bone mineral density, and markers of bone metabolism in men: the Northern Osteoperosis and Obesity (NO2) study. Eur. J. Endocrinol. **158**: 765-770.

Honkatukia, M., Reese, K., Preisinger, R., Tuiskula-Haavisto, M., Weigend, S., Roito, J., Mäki-Tanila, A., and Vilkki ,J. 2005. Fishy taint in chicken eggs is associated with a substitution within a conserved motif of the *FMO3* gene. Genomics **86**: 225-232.

Hood, R.L. and Allen, C.E. 1973. Cellularity of bovine adipose tissue. J. Lipid Res. **14**: 605-610.

Höög, J.O., Hedberg, J.J, Strömberg, P., and Svensson, S. 2001. Mammalian alcohol dehydrogenases – functional and structural characteristics. J. Biomed. Sci. **8:** 71-76.

Imia, T., Jiang, M., Chambon, P., and Metzger, D. 2001. Impaired adipogenesis and lipolysis in the mouse upon selective ablation of the retinoid X receptor a mediated by a

tamoxifen-inducible chimeric Cre recombinase (Cre-ER^{T2}) in adipocytes. PNAS **98**: 224-224.

Institute of Medicine, Food and Nutrition Board. 2001. Vitamin A. Pages 82-161 *in* Dietary reference intakes for vitamin A, vitamin K, arsenic, chromium, copper, iodine, iron, molybdenum, nickel, silicon, vanadium, and zinc. Natl. Acad. Press, Washington, DC.

Iwaki, M., Matsuda, M., Maeda, N., Funahashi, T., Matsuzawa, Y., Makishima, M., and Shimomura, I. 2003. Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. Diabetes **52**: 1655-1663.

Kimchi-Sarfaty, C., Oh, J.M., Kim, I.W., Sauna, Z.E., Calcagno, A.M., Ambudkar, S.V., and Gottesmanm M.M. 2007. A "silent" polymorphism in the *MDR1* gene changes substrate specificity. Science **315**: 525-528.

Kliewer, S.A., Umesono, K., Noonan, D.J., Heyman, R.A., and Evans, R.M. 1992. Convergence of 9-*cis* retinoic acid and peroxisome proliferator signaling pathways through heterodimer formation of their receptors. Nature **358**: 771-774.

Kruk, Z.A., Bottema, C.D.K., Davis, J.J., Siebert, B.D., Harper, G.S., Di,J., and Pitchford, W.S. 2008. Effects of vitamin A on growth performance and carcass quality in steers. Livest. Sci. **19**: 12-21.

Kudla, G., Murray, A.W., Tollervey, D., and Plotkin, J.B. 2009. Coding sequence determinants of gene expression in *Eschrichia coli.* Science **324**: 255-258.

Labrecque, J., Dumas, F., Lacroix, A., and Bhat, P.V. 1995. A novel isoenzyme of aldehyde dehydrogenase specifically involved in the biosynthesis of 9-cis and all-trans retinoic acid. Biochem. J. **305**: 681-684.

Leo, M.A., Iida, S., and Lieber, C.S. 1984. Retinoic acid metabolism by a system reconstituted with cytochrome P-450. Arch. Biochem. Biophys. **234**: 305-312.

Lewin, B. 2008a. The content of the genome. Pages 55-75 *in* Genes IX. Jones and Bartlett Publishers, Sudbury, MA.

Lewin, B. 2008b. RNA splicing and processing. Pages 667-705 *in* Genes IX. Jones and Bartlett Publishers, Sudbury, MA.

MacDonald, P.N. and Ong, D.E. 1988. Evidence of a lecithin:retinol acyltransferase activity in the rat small intestine. J. Biol. Chem. **236**: 12478-12482.

Mata, N.L., Radu, R.A., Clemmons, R.S., and Travis, G.H. 2002. Isomerization and oxidation of vitamin A in cone-dominant retinas: a novel pathway for visual-pigment regeneration. Neuron **36**: 69-80.

McLean, K.L. and Schumtz, S.M. 2009. Associations of melanocortin 1 receptor genotype with growth and carcass traits in beef cattle. Can. J. Anim. Sci, **89**: 295-300.

Michal, J.J., Zhang, Z.W., Gaskins, C.T., and Jiang, Z. 2006. The bovine *fatty acid binding protein 4* gene is significantly associated with marbling and subcutaneous fat depth in Wagyu x Limosin F_2 crosses. Anim. Genet. **34**: 400-402.

Molotkov, A., and Duester, G. 2003. Genetic evidence that retinaldehyde dehydrogenase *ALDH1A1* (*Aldh1A1*) functions downstream of alcohol dehydrogenase *Adh1* in metabolism of retinol to retinoic acid. J. Biol. Chem. **278**: 36085-36090.

Molotkov, A., Deltour, L., Foglio, M.H., Cuenca, A.E., and Duester, G. 2002b. Distinct retinoid metabolic functions for alcohol dehydrogenase genes *Adh1* and *Adh4* in protection against vitamin A toxicity or deficiency revealed in double null mutant mice. J. Biol. Chem. **277:** 13804-13811.

Molotkov, A. Ghyselinck, N.B., Chambon, P., and Duester, G. 2004. Opposing actions of cellular retinol binding-protein and alcohol dehydrogenase control the balance between retinol storage and degradation. Biochem. J. **383**: 295-302.

Molotkov, A., Fan, X., and Duester, G. 2002a. Excessive vitamin A toxicity in mice genetically deficient in ether alcohol dehydrogenase *Adh1* or *Adh3*. Eur. J. Biochem. **269**: 2607-2612.

Montgomery, G.W. and Sise, J.A. 1990. Extraction of DNA from sheep white blood cells. New Zeal. J. Agric. Res. **33**: 437-441.

Napierala, D., Garcia-Rojas, X., Sam, K., Wakui, K., Chen, C., Mendoza-Londono, R., Zhou, G., Zheng, Q., and Lee, B. 2005. Mutations and promoter SNPs in RUNX2, a transcriptional regulator of bone formation. Mol. Genet. Metab. **86**: 257-268.

Napoli, J. 1993. Retinoic acid: its biosynthesis and metabolism. Prog. Nucleic Acid Res. Mol. Biol. **63**: 139-188.

Napoli, J.L. 1996. Retinoic acid biosynthesis and metabolism. FASEB J. **10**: 993-1001.

Napoli, J.L., Boerman, M.H.E.M., Chai, X., Zhai, Y., and Fiorella, P.D. 1995. Enzymes and binding proteins affecting retinoic acid concentrations. J. Steroid Biochem. Molec. Biol. **53**: 497-502.

Nau, H., Chahoud, I., Dencker, L., Lammer, E., and Scott, W. 1994. Teratogenicity of vitamin A and retinoids. Pages 615-664 *in* R. Bloomhoff, ed. Vitamin A in health and disease. Marcel Dekker, Inc., New York, New York.

Negrel, R. 1994. Fat cells cannot divide. Int. J. Obes. **18 (Suppl. 2)**: 88.

NCBI. 2010. dbSNP: Short Genetic Variations. Accessed July 21, 2011. http://www.ncbi.nlm.nih.gov/projects/SNP/index.html

NRC. 1970. Nutrient requirements of beef cattle. 4th rev. ed. Natl. Acad. Press, Washington, DC.

NRC. 1987. Vitamin A. Pages 3-10 i*n* Vitamin tolerance of animals. National Academy Press, Washington, DC.

NRC. 1996. Nutrient requirements of beef cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Oka, A., Iwaki, F., and Dohgo, T. 2004. Effects of vitamin A deficiency on growth hormone secretion and circulating insulin-like growth factor-1 concentration in Japanese Black steers. Anim. Sci, **78:** 31-36.

Oka, A., Maruo, Y., Miki, T., Yamasaki, T., and Saito, T. 1998. Influence of vitamin A on the quality of beef from the Tajima strain of Japanese Black cattle. Meat Sci. **48:** 159-167.

Oota, H., Dunn, C.W., Speed, W.C., Pakstis, A.J., Palmatier, M.A., Kidd, J.R., and Kidd, K.K. 2007. Conservative evolution in duplicated genes of the primate Class I *ADH* cluster. Gene **392:** 64-76.

Parker, R.S. 1996. Absorption, metabolism, and transport of carotenoids. FASEB J. **10**: 542-551.

Perseghin, G., Lattuada, G., De Cobelli, F., Esposito, A., Belloni, E., Canu, T., Ragogna, F., Scifo, P., Del Maschio, A., and Luzi, L. 2007. Serum retinol-binding protein-4, leptin, and adiponectin concentrations are related to ectopic fat accumulation. J. Clin. Endocrinol. Metab. **92:** 4883-4888.

Pey, A.L., Stricher, F., Serrano, L., and Martinez, A. 2007. Predicted effects of missense mutations on native-state stability account for phenotypic outcome in phenylketonuria, a paradigm of misfolding diseases. Am. J. Hum. Genet. **81**: 1006-1024.

Pickworth, C.L., Loerch, S.C., and Fluharty, F.L. 2009. Effects of low vitamin A and D finishing diets on beef cattle carcass quality. J. Anim. Sci. **87 (E-Suppl. 2)**: 519.

Pickworth, C.L., Loerch, S.C., Velleman, S.G., Pate, J.L., Poole, D.H., and Fluharty. F.L. 2011. Adipogenic differentiation state-specific gene expression as related to bovine carcass adiposity. J. Anim. Sci. **89**: 355-366.

Plack, P.A. 1965. Occurrence, absorption and distribution of vitamin A. Proc. Nutr. Soc. **24**: 146-154.

Pugh, K.A. 2007. An evaluation of the corticotropin-releasing hormone and leptin gene SNPs relative to cattle behaviour. M.Sc. Thesis, University of Saskatchewan, Saskatoon, SK.

Pugh, K.A., Stookey, J.M., and Buchanan, F.C. 2011. An evaluation of corticotropin-releasing hormone and leptin SNPs relative to cattle behaviour. Can. J. Anim. Sci. *In press*.

Pyatt, N.A., Berger, L.L., and Nash, T.G. 2005. Effects of vitamin A and restricted intake on performance, carcass characteristics, and serum retinol status in Angus x Simmental feedlot cattle. PAS **21**: 318-331.

Quadro, L., Hamberger, L., Colantuoni, V., Gottesman, M.E., and Blaner, W.S. 2003. Understanding the physiological role of retinol-binding protein in vitamin A metabolism using transgenic and knockout mouse models. Mol. Aspects Med. **24:** 421-430.

Quick, T.C., and Ong, D.E. 1990. Vitamin A metabolism in the human intestinal Caco-2 cell line. Biochemistry **29**: 11116-11123.

Radostits, O.M., Gay, C.C., Blood, D.C., and Hinchcliff, K.W. 2000. Veterinary medicine: a textbook of the diseases of cattle, sheep, pigs, goats, and horses. 9th ed. W.B. Saunders Company Ltd., London, England.

Raghu, P. and Sivakumar, B. 2004. Interactions amongst plasma retinol-binding protein, transthyretin and their ligands: implications in vitamin A homeostasis and transthyretin amyloidosis. Biochim. Biophys. Acta **1703**: 1-9.

Rask, L., Valtersson, C., Anundi, H., Kvist, S., Eriksson. U., Dallner, G., and Peterson, P. A. 1985. Subcellular localization in normal and vitamin A-deficient rat liver of vitamin

A serum transport proteins, albumin, ceruloplasmin and class I major histocompatibility antigens. Exp. Cell Res. **143**: 91-102.

Ren, D., Collingwood, T.N., Rebar, E.J., Wolffem A.P., and Camp, H.S. 2002. PPARγ knockdown by engineered transcription factors: exogenous PPARγ2 but not PPARγ1 reactivates adipogenesis. Genes Dev. **16**: 27-32.

Repa, J.J., Hanson, K.K., and Clagett-Dame, M. 1993. All-*trans*-retinol is a ligand for the retinoic acid receptors. PNAS **90**: 7293-7297.

Ribot, J., Felipe, F., Bonet, M.L., and Palou, A. 2001. Changes in adiposity in response to vitamin A status correlate with changes of PPARγ2 expression. Obes. Res. **9**: 500-509.

Rigtrup, K.M., Kakkad, B., and Ong, D.E. 1994. Purification and partial characterization of retinyl ester hydrolase from brush border of rat small intestine mucosa: probable identity with brush border phospholipase B. Biochemistry **33**: 2661-2666.

Rosen, E.D., Walkey, C.J., Puigserver, P., and, Spiegelman, B.B. 2000. Transcriptional regulation of adipogenesis. Genes Dev. **14**: 1297-1307.

Rozen, S. and Skaletsky, H.J. 2000. Primer3 on the WWW for general users and for biologist programmers. Page 365 *in* S. Krawetz and S. Misener, eds. Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ.

Safonova, I., Aubert, J., Négrel, R., and Ailhaud, G. 1997. Regulation by fatty acids of angiotensinogen gene expression in preadipose cells. Biochem. J. **332**: 235-239.

Savell, J.W., Cross, H.R., and Smithm G.C. 1986. Percentage ether extractable fat and moisture content of beef longissimus muscle as related to USDA marbling score. J. Food Sci. **51**: 838-839.

Schmutz, S.M., Buchanan, F.C., Winkelman-Sim, D.C., Pawlyshyn, V., Plante, Y., McKinnon, J.J., and Fournier, B.P. 2001. Development of the Canadian Beef Reference Herd for gene mapping studies. Theriogenology **55**: 963-972.

Scott, R.E., Florine, D.L., Wille, J.J., and Yun, K. 1982. Coupling of growth arrest and differentiation at a distinct state in the G1 phase of the cell cycle. PNAS **79**: 845-849.

Semba, R.D. 1994. Vitamin A, immunity, and infection. Clin. Infect. Dis. **19**: 489-499.

Senoo, H., Kojima, N., and Sato, M. 2007. Vitamin A-storing cells (stellate cells). Vitam. Horm. **75**: 131-159.

Sidell, N., Famatiga, E., and Golub, S.H. 1981. Augmentation of human thymocyte proliferative responses by retinoic acid. Exp. Cell. Biol. **49**: 239-245.

Sidell, N., Rieber, P., and Golub, S.H. 1984. Immunological aspects of retinoids in humans. I. Analysis of retinoic acid enhancement of thymocyte responses to PHA. Cell Immunol. **87**: 118-125.

Siebert, B.D., Kruk, Z.A., Davis, J., Pitchford, W.S., Harper, G.S., and Bottema, C.D.K. 2006. Effect of low vitamin A status on fat deposition and fatty acid desaturation in beef cattle. Lipids **41:** 365-370.

Smeland, S., Bjerknes, T., Malaba, L., Eskild, W., Norum, K.R., amd Blomhoff, R. 1995. Tissue distribution of the receptor for plasma retinol-binding protein. Biochem. J. **305**: 419-424.

Smith, P.J., Wise, L.S., Berkowitz, R., Wan, C., and Rubin, C.S. 1988. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. J. Biol. Chem. **263**: 9402-9408.

Smith, S.B., Kawachi, H., Choi, C.B., Choi, C.W., Wu, G., Sawyer, J.E. 2009. Cellular regulation of bovine intramuscular adipose tissue development and composition. J. Anim. Sci. **87**: E72-E82.

Sophos, N.A. and Vasiliou, V. 2003. Aldehyde dehydrogenase gene superfamily: the 2002 update. Chem. Biol. Interact. **143-144**: 5-22.

Soprano, D.R. and Blaner, W.S. 1994. Plasma retinol-binding protein. *In* Sporn, M.B., Roberts, A.B., Goodman, D.S. eds. The Retinoids, Biology, Chemistry, and Medicine. Raven Press, New York: pp. 257-282.

Staroscik, A. 2004. Calculator for determining the number of copies of a template. URI Genomics & Sequencing Center. Accessed May 17, 2011. http://www.uri.edu/research/gsc/resources/cdna.html.

Steppan, C.M., Bailey, S.T., Bhat, S., Brown, E.J., Benerjee, R.R., Wright, C.M., Patel, H.R., Ahima, R.S., and Lazar, M.A. 2001. The hormone resistin links obesity to diabetes. Nature **409**: 307-312.

Stewart, M.J., Shean, M.L., Paeper, B.W., and Duester, G. 1991. The role of CCAT/enhancer-binding protein in the differential transcriptional regulation of a family of human liver alcohol dehydrogenase genes. J. Biol. Chem **266**: 11594-11603.

Sundvold, H., Brzozowska, A., and Lien S. 1997. Characterization of bovine peroxisome proliferator-activated receptors γ1 and γ2: genetic mapping and differential expression of the two isoforms. Biochem. Biophys. Res. Commun. **239**: 857-861.

Taimi, M., Helvig, C., Wisniewski, J., Ramshaw, H., White, J., Amad, M., Korczak, B., and Petkovich, M. 2004. A novel human cytochrome P450, CYP26C1, involved in metabolism of 9-*cis* and all-*trans* isomers of retinoic acid. J. Biol. Chem. **279**: 77-85.

Thompson, J.N., Howel, J.McC., and Pitt, G.A.J. 1964. Vitamin A and reproduction in rats. Proc. R. Soc. Lond. B **159**: 510-535.

Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., and Spiegelman, B.M. 1994b. mPPARγ2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. **8**: 1224-1234.

Tontonoz, P., Hu, E., and Spiegelman, B.M. 1994a. Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell **79**: 1147-1156.

Torii, S., Matsui, T., and Yano, H. 1996. Development of intramuscular fat in Wagyu beef cattle depends on adipogenic substances present in serum. Anim. Sci. **63:** 73-78.

Trayhurn, P. and Beattie, J.H. 2001. Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. Proc. Nutr. Soc. **60**: 329-339.

Tsutsumi, C., Okuno, M., Tannous, L., Piantedosi, R., Allan, M., Goodman, D.S., and Blaner, W.S. 1992. Retinoids and retinoid-binding protein expression in rat adipocytes. J. Biol. Chem. **267**: 1805-1810.

Urs, S., Smith, C., Campbell, B., Saxton, A., Taylor, J., Zhang, B., Snoody, J., Voy, B., Moustaid-Moussa, N. 2004. Gene expression profiling in human preadipocytes and adipocytes by microarray analysis. J. Nutr. **134**: 762-770.

Vahlquist, A., Peterson, P.A., and Wibell, L. 1973. Metabolism of the vitamin A transporting protein complex: I. Turnover studies in normal persons and patients with chronic renal failure. Europ. J. Clin. Invest. **3**: 352-362.

van Bennekum, A.M., Fisher, E.A., Blaner, W.S., and Harrison, E.H. 2000. Hydrolysis of retinyl esters by pancreatic triglyceride lipase. Biochemistry **39**: 4900-4906.

Wake, K. 1980. Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs. Int. Rev. Cytol. **66**: 303-353.

Wald, G. 1968. Molecular basis of visual excitation. Science **162**: 230-239.

Wang, W.J., Wang, S.P., Gong, Y.S., Wang, J.Q., and Tan, Z.L. 2007. Effects of vitamin A supplementation on growth performance, carcass characteristics and meat quality in *Limosin* x *Luxi* crossbred steers fed a wheat straw-based diet. Meat Sci. **77:** 450-458.

Wang, Y.H., Bower, N.I., Reverter, A., Tan, S.H., De Jager, N., Wang, R., McWilliam, S.M., Café, L.M., Greenwood, P.L., and Lehnert, S.A. 2009. Gene expression patterns during intramuscular fat development in cattle. J. Anim. Sci. **87**: 119-130.

Wheeler, T.L., Cundiff, L.V., and Koch, R.M. 1994. Effect of marbling degree on beef palatability in *Bos taurus* and *Bos indicus* cattle. J. Anim. Sci. **72**: 3145-3151.

West, K.P. 1994. Vitamin A deficiency: its epidemiology and relation to child mortality and morbidity. Pages 585-614 *in* R. Bloomhoff, ed. Vitamin A in health and disease. Marcel Dekker, Inc., New York, New York.

White, J.A., Beckett-Jones, B., Guo, Y.D., Dilworth, J., Bonasoro, J., Jones, G., and Petkovich, M. 1997. cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450 (CYP26). J. Biol. Chem, **272**: 18538-18541.

White, J.A., Ramshaw, H., Taimi, M., Stangle, W., Zhang, A., Everingham, S., Creighton, S., Tam, S.P., Jones, g., and Petkovich, M. 2000. Identification of the human cytochrome P450, P450RIA-2, which is predominantly expressed in the adult cerebellum and is responsible for all-*trans*-retinoic acid metabolism. PNAS **97**: 6403-6408.

Wolf, G. 2004. The visual cycle of the cone photoreceptors of the retina. Nutr. Rev. **62**: 283-291.

Wolf, G. 2007. Identification of a membrane receptor for retinol-binding protein functioning in the cellular uptake of retinol. Nutr. Rev. **65**: 385-388.

Yeh, W.C., Cao, Z., Classon, M., and McKinight, S.L. 1995. Cascade regulation of terminal adipocyte differentiation by three memebers of the C/EBP family of leucine zipper proteins. Genes Dev. **9**: 168-181.

Yeum, K.J. and Russell, R.M. 2002. Carotenoid bioavailability and bioconversion. Annu. Rev. Nutr. **22**: 483-504.

Zhang, X.K., Lehmann, J., Hoffmann, B., Dawson, M.I., Cameron, J., Graupner, G., Hermann, T., Tran, P., and Pfahl, M. 1992. Homodimer formation of retinoid X receptor induced by 9-*cis* retinoic acid. Nature **358**: 587-591.

Zhang, Y.Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. 1994. Positional cloning of the mouse obese gene and its human homolog. Nature **372**: 425-432.

Ziouzenkova, O., Orasanu, G., Sharlach, M., Akiyama, T.E., Berger, J. P., Viereck, J., Hamilton, J.A., Tang, G., Dolnikowski, G.G., Vogel, S., Duester, G., and Plutzky, J. 2007. Retinaldehyde represses adipogenesis and diet-induced obesity. Nat. Med. **13**: 695-702.

APPENDIX A

Table A.1 Canadian and USDA beef quality grades and their associated marbling scores and minimum intramuscular fat percentage (IMF; Canada Beef Export Federation 2009, Crews *et al*. 2010).

Canada Quality Grade	USDA Quality Grade	USDA Marbling Standard	USDA Marbling Score	Minimum IMF
Prime	Prime	Abundant	10.0	-
Prime	Prime	Moderately Abundant	9.0	-
Prime	Prime	Slightly Abundant	8.0	10.13
AAA	Choice	Moderate	7.0	7.25
AAA	Choice	Modest	6.0	6.72
AAA	Choice	Small	5.0	5.04
AA	Select	Slight	4.0	3.83
A	Standard	Traces	3.0	2.76
-	Standard	Practically Devoid	2.0	-
-	-	Devoid	1.0	-

APPENDIX B

Table B.1 β-carotene content and subsequent vitamin A activity of the major feed ingredients of the backgrounding and finishing diets.

Feed Ingredient	β-Carotene (mg/kg)	Vitamin A (IU/kg)[1]
Barley Straw	0.23	90
Barley Silage	19.60	7840
Barley	0.04	17
Wheat DDGS	0.03	10
Oat Hulls	0.01	4

[1]Calculated as 400 IU/mg β-carotene (NRC 1970)

APPENDIX C

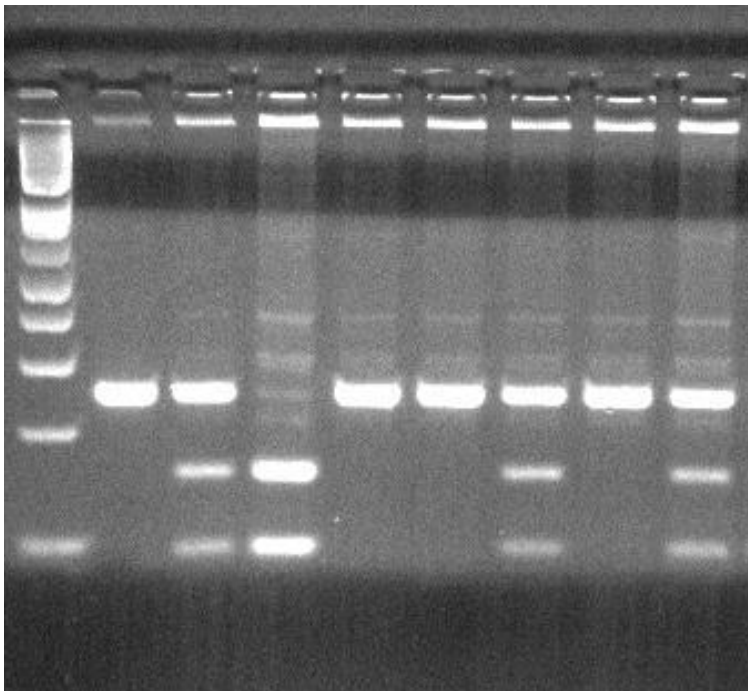

Figure C.1 Agarose gel electrophoresis of the *ADH1C c.-64T>C* PCR-RFLP. Lane 1 is a 1 kb+ ladder, lane 2 is TT (250 bp), lane 3 is CT, and lane 4 is CC (160 + 93 bp).

APPENDIX D

Table D.1 The effect of *ADH1C c.-64T>C* genotype on production and carcass traits in the Pugh (2007) steer population.

	Genotype				
Trait	TT	CT	CC	SEM	P Value
n	195	169	32		
Weight (kg)					
Start of Test	244	242	250	1.0	0.14
End of Test	384	385	393	1.5	0.41
Shipping Weight	428	430	435	1.6	0.51
Final Weight	628	634	638	2.7	0.56
Average Daily Gain (kg/day)					
Backgrounding	1.19	1.21	1.21	0.009	0.55
Final	2.17	2.18	2.17	0.016	0.47
Ultrasound Measurements					
Backfat (mm)	2.2	2.3	2.1	0.07	0.50
Rib-eye Area (cm^2)	64.85	63.82	66.14	0.379	0.27
Warm Carcass Weight (kg)	375.7	377.2	383.1	1.44	0.53
Average Fat (mm)	10	10	9	0.2	0.19
Grade Fat (mm)	8	9	8	0.2	0.12
Rib-eye Area (cm^2)	102	101	105	0.6	0.19
Marbling Score[1]	8	8	8	0.03	0.24
Cutability (%)	61	61	62	0.2	0.17

[1]USDA 10 point scale where 1.0 is Devoid and 10.0 is Abundant

Table D.2 The effect of *ADH1C c.967C>T* genotype on production and carcass traits in the Pugh (2007) steer population.

	Genotype				
Trait	CC	CT	TT	SEM	P Value
n	327	54	1		
Weight (kg)					
Start of Test	243	248	241	1.0	0.21
End of Test	385	388	339	1.5	0.25
Shipping Weight	430	431	380	1.6	0.28
Final Weight	631	636	585	2.7	0.99
Average Daily Gain (kg/day)					
Backgrounding	1.20	1.18	0.85	0.009	0.13
Final	2.17	2.18	2.20	0.016	0.57
Ultrasound Measurements					
Backfat (mm)	2.2	2.4	2.0	0.07	0.64
Rib-eye Area (cm^2)	64.66	64.09	54.07	0.379	0.33
Warm Carcass Weight (kg)	377.1	377.7	329.3	1.44	0.25
Average Fat (mm)	10	10	10	0.2	0.40
Grade Fat (mm)	8	9	7	0.2	0.57
Rib-eye Area (cm^2)	102	101	77	0.6	0.10
Marbling Score[1]	8	8	8	0.03	0.78
Cutability (%)	61	61	62	0.2	0.74

[1]USDA 10 point scale where 1.0 is Devoid and 10.0 is Abundant

Table D.3 The effect of *ALDH1A1 c.*27C>T* genotype on production and carcass traits in the Pugh (2007) steer population.

	Genotype				
Trait	CC	CT	TT	SEM	P Value
n	301	88	4		
Weight (kg)					
Start of Test	243	246	238	1.0	0.33
End of Test	386	382	378	1.5	0.42
Shipping Weight	430	426	419	1.6	0.52
Final Weight	633	624	612	2.7	0.42
Average Daily Gain (kg/day)					
Backgrounding	1.21[a]	1.15[b]	1.16[b]	0.009	**0.01**
Final	2.17	2.15	2.07	0.016	0.65
Ultrasound Measurements					
Backfat (mm)	2.3	2.2	1.8	0.07	0.50
Rib-eye Area (cm^2)	64.41	64.97	64.20	0.379	0.81
Warm Carcass Weight (kg)	376.8	376.4	370.7	1.44	0.95
Average Fat (mm)	10	10	8	0.2	0.62
Grade Fat (mm)	8	8	7	0.2	0.68
Rib-eye Area (cm^2)	102	102	96	0.6	0.89
Marbling Score[1]	8	8	8	0.03	0.71
Cutability (%)	61	61	60	0.2	0.73

[1]USDA 10 point scale where 1.0 is Devoid and 10.0 is Abundant.
[ab]Means within the same row not sharing a common superscript are significantly different ($P<0.05$)

Table D.4 The effect of *ALDH1A1 c.*109A>G* genotype on production and carcass traits in the Pugh (2007) steer population.

	Genotype				
Trait	AA	AG	GG	SEM	P Value
n	314	78	2		
Weight (kg)					
Start of Test	244	244	234	1.0	0.78
End of Test	385	386	361	1.5	0.52
Shipping Weight	430	429	403	1.6	0.51
Final Weight	632	631	617	2.7	0.92
Average Daily Gain (kg/day)					
Backgrounding	1.20	1.20	1.08	0.009	0.67
Final	2.17	2.16	2.29	0.016	0.82
Ultrasound Measurements					
Backfat (mm)	2.3	2.2	2.2	0.07	0.98
Rib-eye Area (cm^2)	64.26	65.42	60.69	0.379	0.37
Warm Carcass Weight (kg)	377.2	375.6	383.1	1.44	0.87
Average Fat (mm)	10	10	11	0.2	0.71
Grade Fat (mm)	8	9	9	0.2	0.80
Rib-eye Area (cm^2)	102	101	89	0.6	0.27
Marbling Score[1]	8	8	8	0.03	0.82
Cutability (%)	61	61	59	0.2	0.63

[1]USDA 10 point scale where 1.0 is Devoid and 10.0 is Abundant

Table D.5 The effect of *PPARγ c.1344G>T* genotype on production and carcass traits in the Pugh (2007) steer population.

	Genotype				
Trait	GG	GT	TT	SEM	P Value
n	348	29	2		
Weight (kg)					
Start of Test	243	244	230	1.0	0.30
End of Test	385	385	360	1.5	0.72
Shipping Weight	430	429	401	1.6	0.71
Final Weight	632	635	598	2.7	0.45
Average Daily Gain (kg/day)					
Backgrounding	1.20	1.18	1.11	0.009	0.98
Final	2.17	2.10	2.46	0.016	0.41
Ultrasound Measerments					
Backfat (mm)	2.3	2.1	2.5	0.07	0.76
Rib-eye Area (cm^2)	64.48	64.93	63.01	0.379	0.87
Warm Carcass Weight (kg)	377.3	378.4	352.8	1.44	0.46
Average Fat (mm)	10	10	9	0.2	0.30
Grade Fat (mm)	8	8	7	0.2	0.51
Rib-eye Area (cm^2)	102	102	100	0.6	0.48
Marbling Score[1]	8	8	8	0.03	0.21
Cutability (%)	61	61	61	0.2	0.45

[1]USDA 10 point scale where 1.0 is Devoid and 10.0 is Abundant

APPENDIX E

Plant and Animal Genome XIX Conference, Jan 15-19, 2011, San Diego, CA.

Interactions between vitamin A supplementation and a novel mutation in *ADH1C* affect intramuscular fat content in finishing steers

A.K. Ward and F.C. Buchanan

Studies have demonstrated that vitamin A supplementation can impact intramuscular fat (IMF) deposition in cattle. The purpose of this study was to examine if a mutation in *alcohol dehydrogenase 1C* (*ADH1C*), which metabolizes retinol to retinaldehyde, interacts with vitamin A supplementation to affect IMF content in cattle. An initial population 450 Black Angus-cross steers were genotyped at *ADH1C c.-64T>C*. Subsequently 50 TT, 50 CT, and 30 CC animals were randomly allocated to the feeding trial. All animals were backgrounded on a β-carotene deficient diet for 3 months to deplete liver vitamin A stores (confirmed by liver biopsy). The steers were finished on a standard barley-based finishing ration that did not contain any supplemental vitamin A. In a 3 x 2 (genotype x vitamin A level) factorial design, steers received a monthly bolus of either 0 or 750,000 IU vitamin A throughout finishing. Carcasses were evaluated for marbling score according to USDA standards and a sample of the *longissimus dorsi* muscle was analyzed for IMF content. Marbling score and IMF were significantly higher in the steers receiving 0 IU vitamin A ($P<0.05$). There was a significant interaction between *ADH1C* genotype and vitamin A level on IMF; TT steers fed 0 IU vitamin A had nearly 25% more IMF than those supplemented with 750,000 IU vitamin A. Additionally, at 0 IU vitamin A supplementation IMF was 23% higher in TT steers than CC steers. In conclusion, an interaction between *ADH1C c.-64T>C* and vitamin A was found to influence marbling in finishing steers.

APPENDIX F

Plant and Animal Genome XIX Conference, Jan 15-19, 2011, San Diego, CA.

P530

Interactions between vitamin A supplementation and a novel mutation in *ADH1C* affect intramuscular fat content in finishing steers

Alison K. Ward and Fiona C. Buchanan

Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, SK.

Introduction

- The recommended vitamin A level for beef steers is 2200 IU/kg DM[1] (~25,000 IU/day)
- Associations have been found between vitamin A restriction and marbling in beef cattle[2]
- Alcohol dehydrogenase 1C (ADH1C) oxidizes retinol (ROL) to retinaldehyde (RALD)[3]
  - RALD is oxidized to retinoic acid (RA)[4]

ROL —ADH1C→ RALD ⟶ RA

- RA binds to nuclear receptors RXR and RAR, which activate genes involved in adipogenesis[5,6]
- RALD inhibits RXR and RAR[6]
- The purpose of this study was to examine the effects of vitamin A supplementation and a mutation in *ADH1C* on marbling in beef steers

Materials & Methods

- 450 Black Angus-cross steers were genotyped at *ADH1C c.-64T>C* by PCR-RFLP
- 50 TT, 50 CT, and 30 CC steers were randomly allocated to the feeding trial
- Backgrounded on a β-carotene deficient diet for 3 months
- Finished for 5 months on a barley-based ration with no supplemental vitamin A
- Allocated to one of two treatment groups for the finishing period:
  - Low (0 IU vitamin A/month)
  - Standard (750,000 IU vitamin A/month)
- Treatments delivered monthly by oral bolus
- Slaughtered over a period of 2 weeks
- Carcasses were graded according to Canadian standards and assigned a USDA marbling score
- Intramuscular fat (IMF) was measured by petroleum ether extraction[7]
- Data were analyzed in a 3 x 2 (genotype x vitamin A level) factorial design using the mixed procedure of SAS 9.2[8]
- Significance was set at P<0.05

Results

Table 1. The main effects of *ADH1C c.-64T>C* genotype, vitamin A treatment, and their interaction on production and carcass traits. Bolded values are significant (P<0.05).

Variable	ADH1C	Vitamin A	Interaction
Average Daily Gain	0.3390	0.6328	0.7268
Ship Weight	0.3610	0.3499	0.6941
Hot Carcass Weight	0.7674	0.6873	0.8963
Dressing %	0.2187	0.2648	0.5582
Grade Fat	0.1227	0.7556	0.9817
Rib-eye Area	0.4345	0.4472	0.4816
Lean Yield	0.3257	0.4520	0.5236
Marbling Score	0.5369	**0.0481**	0.1622
Intramuscular Fat	0.5162	**0.0231**	**0.0105**

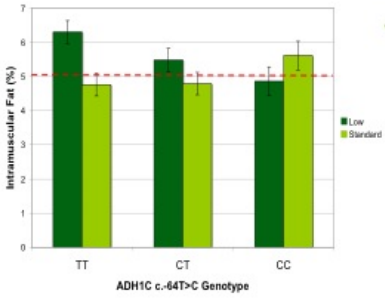

Figure 1. The intramuscular fat content of the *longissimus dorsi* of TT, CT, and CC steers at *ADH1C c.-64T>C* administered 0 IU/month vitamin A (Low) or 750,000 IU/month vitamin A (Standard). Treatments not sharing a common superscript are significantly different (P<0.05). Dashed line demarcates the minimum IMF content required for USDA Choice quality grade[9].

- Allele frequencies were 0.70 and 0.30 for T and C respectively
- IMF is significantly associated with the interaction between *ADH1C* genotype and vitamin A supplementation level
- TT steers had nearly 25% greater IMF in the low vitamin A treatment than the standard treatment
- TT steers had 23% greater IMF than CC steers in the low vitamin A treatment

Discussion

- RALD inhibits adipogenic gene activation[6]
  - Less retinol → less RALD → less inhibition
  - Vitamin A restriction could therefore increase activation of adipogenic genes in TT steers, resulting in greater IMF content
- The C allele removes a potential binding motif for the transcription factor C/EBPα[10], possibly reducing *ADH1C* expression
- CC steers might produce adequate levels of RALD when retinol is plentiful but inadequate levels when retinol is limited
  - RALD levels could be low enough that insufficient RA is available to maximize activation of adipogenic genes

Conclusion

- Vitamin A restriction was associated with increased IMF in TT steers but had no significant effect in CT or CC steers
- Vitamin A restriction could potentially increase the average quality grade in TT steers from USDA Select (Canada AA) to Choice (AAA)[9]
- Reduced vitamin A supplementation and *ADH1C c.-64T>C* marker-assisted management could be used to increase marbling in feedlot steers

References

1. NRC. 1996. Nutrient requirements of beef cattle. 7th ed. National Academy Press, Washington, D.C.
2. Gorocica-Buenfil, M.A. *et al.* 2007. J. Anim. Sci. 85: 2243-2255.
3. Molotkov, A. *et al.* 2002. J. Biol. Chem. 269: 2607-2612.
4. Molotkov, A. and Duester, G. 2003. J. Biol. Chem. 278: 36085-36090.
5. Heyman, R.A. *et al.* 1992. Cell 68: 397-406.
6. Zlouzenkova, O. *et al.* 2007. Nat. Med. 13: 695-702.
7. AOAC. 1990. Official methods of analysis. 15th ed. Association of Official Analytical Chemists, Arlington, VA.
8. SAS 9.2. 2008. SAS Institute Inc. Cary, NC.
9. Hohenboken, W.D., ed. 2002. Guidelines for uniform beef improvement programs. 8th ed. Beef Improvement Federation
10. Chekmenev, D.S. *et al.* 2005. Nucleic Acids Res. 33: W432-W437.

Acknowledgements

ALMA

POUND-MAKER